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Putrescine biosensor based on putrescine oxidase from *Kocuria rosea*

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ABSTRACT

The novel putrescine oxidase based amperometric biosensor selectively measures putrescine, which can be considered as an indicator of microbial spoilage. Putrescine oxidase (PUOX, EC 1.4.3.10) was isolated from *Kocuria rosea* (*Micrococcus rubens*) by an improved and simplified purification process. Cells were grown on brain heart infusion medium supplemented with putrescine. Cell-free extract was prepared in Tris buffer (pH 8.0) by Bead-beater. A newly elaborated step based on three-phase partitioning (TPP) was applied in the purification protocol of PUOX.

The purified enzyme was immobilized on the surface of a spectroscopic graphite electrode in redox hydrogel with horseradish peroxidase, Os mediator and poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) as crosslinking agent. This modified working electrode was used in wall-jet type amperometric cell together with the Ag/AgCl (0.1 M KCl) reference electrode and a platinum wire as auxiliary electrode in flow injection analysis system (FIA). Hydrogel composition, pH and potential dependence were studied. Optimal working conditions were 0.45 mL min^{-1} flow rate of phosphate buffer (66 mM, pH 8.0) and +50 mV polarizing potential vs. Ag/AgCl. The linear measuring range of the method was 0.01–0.25 mM putrescine, while the detection limit was 5 μM. Beer samples were investigated by the putrescine biosensor and the results were compared by those of HPLC reference method.

1. Introduction

Putrescine belongs to the group of low molecular weight organic bases of biological importance referred to biogenic amines. This group of biologically active natural compounds is mainly formed by microbial decarboxylation of amino acids. The diamine putrescine is produced from ornithine and is a precursor for polyamine formation. Although intake of dietary polyamines has been known as an important factor of health and disease for years, data on polyamine content in foods are limited and dispersed in the literature [1]. High putrescine contents are usually connected with high activity of several groups of bacteria, mainly *Enterobacteriaceae* and *Clostridium* spp. [2]. Therefore putrescine content in food can be considered as a freshness marker and be used as an indicator of microbial spoilage [3]. The most frequently investigated foods are cheeses, fish and meat products, but beer has been reported also as a possible health risk for some consumers due to its biogenic amine content and the relatively high amount consumed [4].

The analytical methods used for the determination of biogenic amines are mainly based on chromatographic methods: thin layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). Among them HPLC is the most often used for food analysis. The majority of assays apply fluorometric or spectroscopic detection with pre- or post-column derivatization techniques [5]. Contrary to the above mentioned methods, biosensors offer simple, rapid and cost-effective solution for the determination of biogenic amines, especially for putrescine.

Biosensors for biogenic amine determination are based on amine oxidases, mainly plant diamine oxidases [6–15]. As the substrate specificity of these enzymes is broad, they are suitable for the measurement of total biogenic amine content. Selective determination of spoilage indicator amines, like putrescine, offers an alternative approach of food quality control by using selective enzymes like putrescine oxidase from *Kocuria rosea* (*M. rubens*). The catalytic activity of putrescine oxidase toward mono- and diamines has already been investigated. It was reported, that only putrescine could be oxidized rapidly, while cadaverine and spermidine were converted at a much slower rate compared to putrescine [16,17]. Different immobilization techniques have been used for putrescine biosensors. Enzyme reactors were investigated by covalently binding the enzyme onto controlled pore aminopropyl glass beads [18,19] or on chitosan porous beads [20]. Other techniques include the immobilization of the enzyme on the surface of working electrodes by cross-linking with glutaraldehyde in case of silanized screen-printed platinum electrodes [21], glassy carbon electrodes

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[19] or by deposition on multiwalled carbon nanotube (MWCNT) modified glassy carbon electrode [22].

Many of the presented putrescine biosensors belong to the first generation biosensors, that are based on the determination of hydrogen peroxide produced in the enzyme reaction [18,20,21]. A POUX-based electrode was also tested with different artificial mediators such as ferrocene derivatives. None of these mediators were able to shuttle the electrons between the active site of PUOX and the electrode surface [19]. However this type of diffusional mediator could be successfully used for the detection of putrescine at +50 mV potential, when PUOX is coupled with HRP [19]. Nowadays much effort is made toward preparing biosensors based on direct electron transfer (DET) between the active center of enzymes and the electrode. This phenomenon is extremely difficult in case of PUOX, because the FAD moiety is deeply embedded within the protein [22]. However, there are attempts to create electron bridge between glassy carbon electrode and FAD group of the PUOX enzyme by applying multiwalled carbon nanotubes (MWCNTs) [22]. The most promising way to achieve DET is coupling PUOX enzyme with peroxidase, which is one of the enzymes that show DET ability [23].

These putrescine biosensors were mainly applied for monitoring the freshness or spoilage of fish [18,19,21] and chicken [20]. Putrescine oxidase based biosensors were also applied for the determination of putrescine level in blood [24,25].

The isolation and characterization of putrescine oxidase enzyme from *M. rubens* based on ammonium sulfate fractionation and heat treatment steps followed by DEAE Sephadex chromatography was reported in the 70s [17].

The aim of our present work was to develop a biosensor method for rapid determination of putrescine in food samples e.g. beer. The amperometric biosensor is based on putrescine oxidase isolated from *K. rosea*.

This work reports for the first time putrescine oxidase (PUOX), horseradish peroxidase (HRP) and Os-mediator (PVI7-dme-Os) modified carbon electrodes for putrescine determination. The combined application of the putrescine oxidase and horseradish peroxidase enzymes resulted in reduced operational potential, thus contributes to surmounting obstacles concerning hydrogen peroxide detection, such as high background currents and presence of disturbing signals. Specific and novel feature of our approach was the fact, that the enzymes were immobilized on the electrode surface in redox hydrogel with Os complex (mediator), forming a cross-linked, three-dimensional, polymeric matrix, referred to as "wired" enzyme electrodes. This type of redox hydrogel makes possible electron diffusion for charge transfer. It is also permeable to water-soluble substrates and products of the enzyme reactions; therefore it is applicable for mediated reagentless biosensor [26].

In this paper we also report a new purification procedure for the enzyme, in which ammonium sulfate precipitation was replaced by three-phase partitioning (TPP). TPP is a powerful separation technique based on joint use of a salt and an organic solvent miscible with water to precipitate proteins from aqueous solutions. In the presence of ammonium sulfate at concentrations higher than 20% relative saturation, the t-butanol–water mixture separates into two phases and the precipitated proteins collect at the interface. This technique is useful both "upstream" for liter scale precipitation of enzymes and "downstream" for semi micro, milliliter volume scale [27–29].

2. Material and methods

2.1. Bacteria

A culture of *K. rosea* (*M. rubens*) K22 was obtained from the Microbiology Department of the Eötvös Loránd University (Budapest, Hungary) and was maintained on nutrient agar slants.

2.2. Chemicals

Peroxidase from horseradish (HRP) (EC 1.11.1.7, 1100 U mg^{-1} solid) was used as received from Sigma–Aldrich (St. Louis, MO, USA). Putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, spermine tetrahydrochloride, spermidine trihydrochloride, tyramine hydrochloride, tryptamine hydrochloride, flavin adenine dinucleotide disodium salt hydrate (FAD), Tris(hydroxymethyl)aminomethane (TRIS), ammonium sulfate, dansyl chloride (DCl) and albumin from bovine serum (BSA) were obtained from Sigma–Aldrich. Bradford Reagent was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA), DEAE-Cellulose from Reanal (Budapest, Hungary), and Express Ion D from Whatman (Maidstone, Kent, UK). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) used as crosslinking agent was from Polysciences (Warrington, PA, USA). The Os-mediator (PVI7-dme-Os), a complex of poly(vinylimidazole) with Os(4,4′ dimethylbipyridine)$_2$Cl, was synthesized according to a previously published protocol [30], and was kindly provided by Csöregi (Lund University, Lund, Sweden).

Water purified with a Milli-Q system (Millipore, Badford, MA, USA) was used.

2.3. Beer samples

Beer samples were purchased in a local shop and stored at 4 °C before measurement. Beers were filtered and degassed by ultrasonic treatment (20 min).

2.4. Apparatus for biosensor measurement

The biosensor works in flow injection analysis system (FIA) using QuadStat 164 potentiostat and e-corder A/D converter (eDAQ Pty Ltd., Denistone East, Australia) coupled with a wall-jet type amperometric cell in three electrode arrangement with a modified graphite working electrode, a Ag/AgCl (0.1 M KCl) reference electrode and a platinum wire as auxiliary electrode. Chart software (eDAQ Pty Ltd., Denistone East, Australia) was used for data acquisition and processing. The constant buffer flow was maintained by peristaltic pump Minipuls 4 (Gilson, Villiers le Bel, France). The samples were injected with a Rheodyne 7725i type (Rohnert Park, CA, USA) manual injector using 20 μL sample loop.

2.5. Enzyme electrode preparation

Graphite electrodes (i.d. 3.05 mm, type RW 001, Ringsdorff Werke, Bonn, Germany) were wet polished on emery paper and thoroughly washed with distilled water before modification. Premixed hydrogel was prepared using the following stock solutions: putrescine oxidase (2 mg mL^{-1} = 15.8 U mL^{-1}), peroxidase (10 mg mL^{-1} = 11,000 U mL^{-1}), PVI7-dme-Os (5 mg mL^{-1}) and PEGDGE (3.33 mg mL^{-1}, freshly prepared in high purity deionized water, and used within 15 min) as detailed later. The hydrogel was placed on the electrode surface. The modified electrodes were dried at room temperature, and stored overnight in the refrigerator before using.

All presented results are the mean of at least three identically prepared electrodes, if not otherwise mentioned.

2.6. HPLC measurements

HPLC measurements were performed with Agilent 1200 Series instrument (Agilent, Santa Clara, CA, USA) comprising a high pressure gradient pump, an autosampler and a DAD detector, and equipped with ChemStation software. A reverse phase silica column (Prodigy 5 μm ODS-3V, 250 mm × 4.60 mm, Phenomenex, Torrance, CA, USA) was used. Precolumn derivatization with dansyl chloride was applied, and the eluted dansylated amines were detected at 254 nm [31]. The gradient elution program using (A) water and (B) acetonitrile with a flow-rate of 0.8 mL min^{-1} was the following: 0–1 min (65%B); 1–13 min (80 → 90%B); 16–23 min (100%B); 24–35 min (65%B).

For derivatization 0.5 mL of DCl reagent (20 mg mL^{-1} in acetone) was added to 0.25 mL of sample, pH of the solution was set to 11.0 and the mixture was incubated in dark for 60 min at 40 °C. In order to eliminate the excess of DCl, samples were treated with 0.05 mL of concentrated ammonia solution, vortexed for 1 min and incubated in darkness for 15 min at ambient temperature. Before injection the pH of the sample was controlled, 0.540 mL acetonitrile was added and the insoluble particles were removed by centrifugation (10 min, 14,000 × *g*). Standard samples containing biogenic amines in different concentrations (1–50 mg mL^{-1} in water) were used for calibration.

2.7. Spectrophotometric measurements

Spectrophotometric measurements were made by Metertech UV/VIS SP8001 (Metertech Inc., Taipei, Taiwan) spectrophotometer with 1.0 cm light path.

2.7.1. Enzyme assay

Putrescine oxidase activity was assayed spectrophotometrically at 510 nm using 4-aminoantipyrine that gives quinoneimine dye in reaction with hydrogen peroxide produced. Assays were carried out at 30 °C, in 0.04 M Tris–HCl (pH 8.0) buffer. The assay mixture contained 1.58 mM putrescine, 0.47 mM 4-aminoantipyrine, 0.23 mM

2,4-dichlorophenol and 3.2 U mL^{-1} peroxidase in 0.04 M Tris–HCl (pH 8.0) buffer, respectively. One unit putrescine oxidase catalyses the formation of one micromole hydrogen peroxide (half a micromole quinoneimine dye) per minute at 30 °C/min.

2.7.2. Protein measurements

The protein content of the eluates was followed by measuring absorbance at 280 nm. The protein concentration of the samples was measured by the Bradford method [32] at 595 nm.

2.8. Isolation of putrescine oxidase enzyme from K. rosea

2.8.1. Enzyme extract preparation

K. rosea (*M. rubens*) was maintained on nutrient agar slants, and was grown on Difco Brain Heart agar (15 g L^{-1}) supplemented with 0.02% putrescine dihydrochloride (1.25 mM) in culture dishes. Cells were harvested after 5–7 day storage at 37 °C. Bacterial cells were suspended in 0.05 M Tris–HCl buffer (pH 8.0) to a concentration of about 0.2 g wet weight per mL. One part of the cold suspension and one part of ice-cold, 0.1 mm diameter beads were mixed and disrupted by BeadBeater (BioSpec Products Inc., Bartlesville, OK, USA). Cooling jacket filled with crushed ice and water was used to minimize warming. The effect of BeadBeater operation time was studied under the applied conditions (data are not shown) the optimal operation time was found to be 2 min. Cellular debris and beads were removed from the homogenate by centrifugation for 15 min at 15,000 × *g* at 4 °C and the cell-free extract was used for enzyme purification.

2.8.2. Enzyme extract purification

A new purification protocol was elaborated with the successive application of TPP and ion exchange chromatography. The optimized conditions are summarized in the following section.

2.8.3. Three-phase partitioning (TPP)

The cell-free extract was purified with TPP as follows: the solution was brought to 66% relative saturation with addition of two volume of saturated $(NH_4)_2SO_4$ solution to one volume of protein solution, than t-butanol was added to the mixture up to 30% of its final volume. The mixture was shaken rigorously. After waiting 5 min it was centrifuged (3 min, 13,000 × *g*) to reduce salt and alcohol content in the midlayer and to facilitate its removal from the system at the same time. The midlayer was collected and washed briefly with ice-cold distilled water, than 0.05 M Tris–HCl buffer (pH 8.0) was added and the suspension was stirred for 1 h at 4 °C. It was cleared by centrifugation (3 min, 13,000 × *g*). Most *Kocuria* proteins lost their solubility during TPP process and did not redissolve in Tris buffer, one of the exceptions is PUOX.

Putrescine oxidase contains one FAD moiety per enzyme molecule, but this cofactor can dissociate reversibly during ammonium sulfate treatment. To eliminate this effect, after TPP the supernatant was reactivated by incubation with FAD added in excess amount.

2.8.4. DEAE-cellulose chromatography

For ion exchange chromatography of PUOX batch adsorption and column chromatography were tested.

2.8.4.1. Batch technique. The FAD treated supernatant was added to the DEAE Cellulose equilibrated with 0.05 M Tris–HCl buffer (pH 8.0), and it was stirred for 10–30 min. The adsorption of the enzyme was controlled by enzyme activity measurement. After binding the bulk of enzyme, the suspension was filtered under suction. A fraction of proteins was removed from the DEAE cellulose by washing with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.25 M NaCl. Putrescine oxidase was eluted from DEAE cellulose with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.75 M NaCl.

2.8.4.2. Column chromatography. The FAD treated supernatant was applied to an Express Ion D column (10 cm length, 15 mm i.d.) equilibrated with 0.025 M Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl. The column was washed with buffer containing 0.3 M NaCl. Putrescine oxidase was eluted by linear salt gradient 0.3–0.6 M NaCl in 0.025 M Tris–HCl buffer (pH 8.0). Absorbance of the eluate was followed in a quartz flow-through cuvette at 280 nm by UV/VIS Spectrophotometer (Jasco 7850, Tokyo, Japan). The active fractions were concentrated with Centricon Plus-80 ultrafiltration devices (Millipore, Billerica, MA, USA).

3. Results and discussion

3.1. Enzyme isolation

One of our aims was to develop a protocol for the isolation and purification of high purity putrescine oxidase (PUOX) suitable for biosensor application, from the bacteria *K. rosea*. In the purification method reported by DeSa [17] ammonium sulfate fractionations and heat treatment were followed by DEAE Sephadex chromatography. This procedure was replaced with one TPP step and the subsequent ion exchange chromatography, as detailed in the Experimental section. The purification process became approx. 1.5 days shorter with these modifications. After TPP, PUOX was found in the midlayer between the lower aqueous and the upper organic phase. After rapid washing with cold distilled water, the separated midlayer was dissolved in the start buffer used for ion exchange chromatography (0.05 M Tris–HCl buffer, pH 8.0). The redissolved proteins were further purified with ion exchange chromatography applying both batch and column chromatographic setup.

In the batch method 6.2 U of PUOX enzymes was obtained from 9.5 g bacteria, resulting in an overall yield of 62%, and the specific activity of the end product was 1.7 U mg^{-1}, corresponding to a medium specific activity.

During the column chromatographic run, protein fractions, PUOX I and PUOX II, were found to have enzyme activity. PUOX I was eluted in 0.45 M NaCl (26 min), while PUOX II in 0.5 M NaCl (63 min). These fractions were concentrated with Centricon YM-10 ultrafiltration devices (Millipore, Billerica, MA, USA). Enzyme activities of PUOX I and PUOX II were 25 U mL^{-1} (1.1 U mg^{-1}), and 5.5 U mL^{-1} (7.9 U mg^{-1}), respectively. The molecular mass of the enzyme was found at approximately 90–100 kDa by SDS-PAGE which corresponds to the dimeric flavoprotein consisting of ~50 kDa subunits and containing one FAD moiety. This is in agreement with molecular mass reported by DeSa [17] and by Ishizuka et al. [33]

The PUOX II enzyme fraction yielded significantly higher specific activity, so this product was used for biosensor development.

3.2. Biosensor optimization

The purified enzyme (PUOX II) was co-immobilized on the surface of a graphite electrode with horseradish peroxidase, Os mediator (PVI7-dme-Os) and PEGDGE as crosslinker. This modified working electrode was employed in a wall-jet amperometric cell together with a Ag/AgCl (0.1 M KCl) and a platinum electrode served as reference and auxiliary electrode, respectively and applied in FIA system. The measuring parameters of the biosensor were studied and optimized. All procedures were performed using putrescine as substrate.

3.2.1. Effect of applied polarization potential

The signal of the putrescine biosensor was studied at different putrescine concentrations (0.01 and 1 mM) as a function of polarization. The potential was found to be optimal between −50 mV and +50 mV. Further studies were carried out at +50 mV.

3.2.2. Effect of electrode composition

Working electrodes with different composition were prepared and their responses to putrescine standard solutions (0.01 and 1 mM) were studied in Tris–HCl buffer (pH 8.0) at +50 mV polarization potential. The amounts of the enzymes (putrescine oxidase and horseradish peroxidase) and Os mediator in the hydrogel used for electrode modification were changed systematically. Fig. 1 displays results obtained with the surface modified electrodes.

Significantly increased amperometric signal could be observed with putrescine solutions, when comparing the enzyme electrodes with bare graphite (A) electrodes. The Os mediator co-immobilized with PUOX on the enzyme electrode surface enhances the signal by app. 70% (electrodes G and F) measured at +50 mV polarization potential. HRP content showed similar effect; signals on electrode E and F were almost the same. Some more increase in the measured signal could be detected in case of their combined application (electrode D). The effect of putrescine oxidase (PUOX) content was also studied by varying from 4.2 to 7.8 mU in case of the mediated bienzyme electrode (B–D). The amperometric signal did not show a

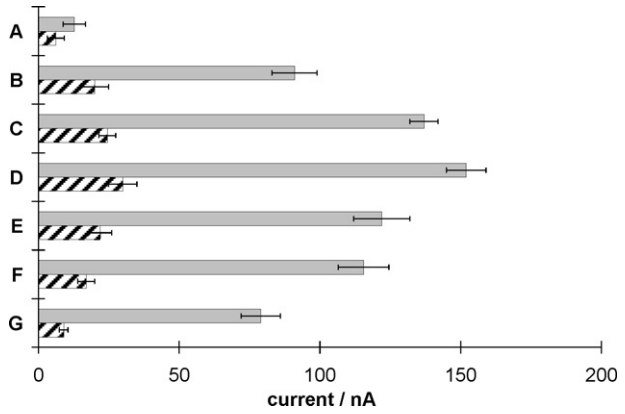

Fig. 1. Response of electrodes with different hydrogel composition (putrescine: 1 mM (gray), 0.1 mM (striped), +50 mV, 0.05 M Tris–HCl buffer, pH 8.0). Electrode compositions: (A) unmodified electrode; (B) 4.2 mU PUOX, 16 U HRP, 5 μg Os mediator, 5 μg PEGDGE; (C) 5.6 mU PUOX, 16 U HRP, 5 μg Os mediator, 5 μg PEGDGE; (D) 7.8 mU PUOX, 16 U HRP, 5 μg Os mediator, 5 μg PEGDGE; (E) 7.8 mU PUOX, 16 U HRP, 5 μg PEGDGE; (F) 7.8 mU PUOX, 5 μg Os mediator, 5 μg PEGDGE; (G) 7.8 mU PUOX, 5 μg PEGDGE.

proportional rise to the increase of enzyme load per electrode, and the stability of the electrodes was not improved either. Based on these results, electrode C (modified with 5.6 mU putrescine oxidase, 16 U peroxidase, 5 μg Os mediator and 5 μg PEGDGE) was selected for further work.

3.2.3. Effect of pH, composition and concentration of buffer

The effect of pH on the biosensor response was studied in the range pH 6.4–9.2 at different putrescine concentrations (0.005–1 mM). The highest signal responses were detected at pH 8.0–8.6 (Fig. 2). Although the signals increased slightly up to pH 8.5, the best linearity and stability of the measured signal could be observed at pH 8.0, so this pH was used during measurements.

Studies were carried out in the 0.025–0.200 M concentration range of phosphate and Tris–HCl buffers (pH 8.0). The optimum concentration of both buffers was 0.050–0.075 M. The highest signal and the best linearity were observed using phosphate buffer (0.066 M, pH 8.0).

3.2.4. The effect of flow rate

The effect of flow rate on the biosensor response was studied in the range 0.15–0.85 mL min^{-1} using phosphate buffer (0.100 M, pH 8.0), and the highest responses and linear calibration curves were obtained applying 0.4 mL min^{-1} flow rate.

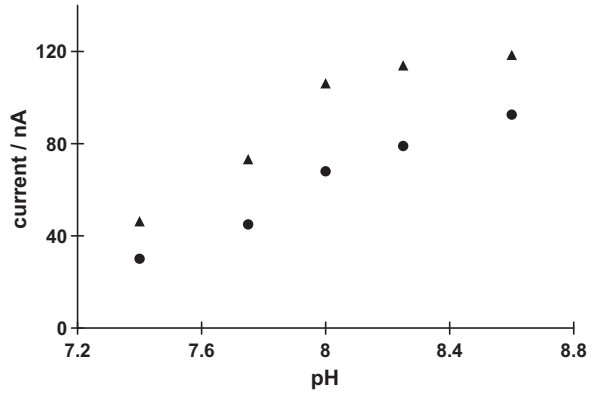

Fig. 2. Dependence of putrescine biosensor response on pH (putrescine: 0.5 mM (▲), 0.25 mM (•), electrode C, +50 mV vs. Ag/AgCl, 0.05 M Tris–HCl buffer).

Table 1
Substrate specificity of PUOX biosensor.

Biogenic amine	Relative activities (%)
Putrescine	100
Cadaverine	10.7
Tryptamine	5.2
Spermidine	5.0
Tyramine	2.0
Histamine	1.7

3.3. Biosensor selectivity

The selectivity of the developed biosensor method was tested. The most abundant mono-, di- and polyamines with potential importance in food quality and safety, namely cadaverine, histamine, tyramine, tryptamine, spermine and spermidine, were chosen for selectivity studies. Signals of these amines were investigated at concentrations of 0.1 and 1 mM under the optimized working conditions. Immobilized PUOX almost selectively reacted with its natural substrate, putrescine. Cadaverine, tryptamine, tyramine, spermidine and histamine produced much smaller responses (Table 1).

These results agree with those published previously for free putrescine oxidase [16,34]. As our putrescine biosensor showed excellent substrate specificity, it could be used for selective determination of putrescine in different food products.

3.4. Biosensor characteristics

Fig. 3 shows a typical standard calibration curve for putrescine obtained with PUOX-based biosensor under optimal working conditions. The linear measuring range was detected in the concentration range of 0.01–0.25 mM, and a detection limit (calculated as three times the noise) of 5 μM.

The operating and storage stability of the putrescine sensor was studied as well. The freshly prepared enzyme electrodes could be stored in the refrigerator for 7–10 days without significant loss of activity. The PUOX modified enzyme electrode could be used for 120–150 sample injections without considerable decrease in the response. When the electrode was stored at 4 °C in phosphate buffer (pH 8.0) overnight between measurements, a decrease in the current response up to 40% of the original value was observed.

3.5. Determination of putrescine content in real food (beer) samples

Beer has been commonly reported to be a health risk for some sensitive consumers due to its biogenic amine content [4], therefore their analysis is important for food safety [35,36].

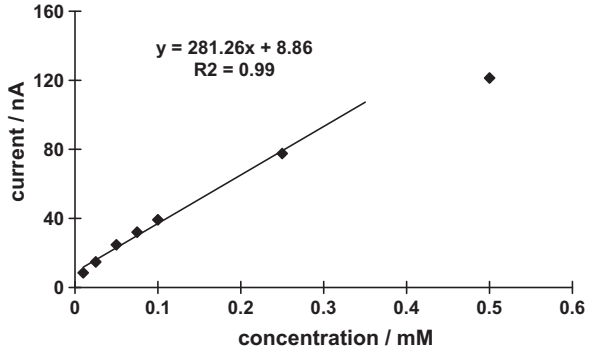

Fig. 3. Calibration curve for putrescine with putrescine oxidase-based biosensor (+50 mV, 0.45 mL min^{-1} 0.066 M phosphate buffer, pH 8.0).

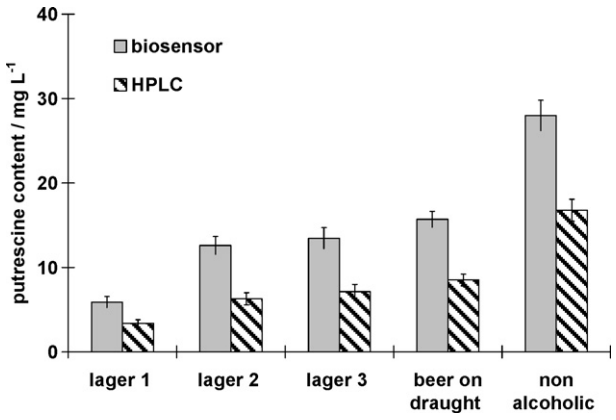

Fig. 4. Putrescine contents of beer samples measured by putrescine biosensor (gray) and by HPLC (striped).

The developed putrescine biosensor was applied for the determination of putrescine content of some beer samples. Since beers have a water-based sample matrix, very little sample preparation is required. Samples with different dilution (3–50 times) were tested. It was concluded, that samples diluted 10–20 times were best fitted to the measuring range. Beer samples were analyzed by standard addition method as well. They were spiked with 0–0.1 mM putrescine and 85% recovery was obtained.

The putrescine concentrations in beer samples studied were in the range of 1.5–15.2 mg L^{-1}, which is reported in European beers [37]. In case of non-alcoholic beer higher putrescine content was detected, it is over the above mentioned interval. However, it cannot be considered extremely high, as similar data were reported in case of Czech beers [35]. Comparable putrescine contents were obtained for lager beers from the same brewery (lager 2, lager 3 and beer on draught). Analog results were presented [35] comparing the biogenic amine levels of beers from a brewery in different batches.

For comparison, biogenic amine contents of selected beers were examined by HPLC, as well (Fig. 4). However the putrescine contents determined by HPLC were significantly lower than those measured with the biosensor. This was probably due to the limited enzyme activity to other biogenic amines present in beer samples, but the RSD was 0.98 between the two different methods.

The results of this work demonstrate that this is a simple, reliable and rapid method for the determination of putrescine in food samples.

4. Conclusions

The aim of our present work was to develop a biosensor based on enzyme modified carbon electrode for rapid determination of putrescine content in food samples, for food quality control purposes. Putrescine oxidase (EC 1.4.3.10) enzyme applied was isolated from *K. rosea* (*M. rubens*) by an improved and simplified purification procedure. The new purification protocol was elaborated with the successive application of three-phase partitioning and ion exchange chromatography. Immobilization of the purified putrescine oxidase enzyme on the surface of a graphite electrode with horseradish peroxidase and Os mediator (PVI7-dme-Os) in a redox hydrogel might be regarded as a novel approach. This "wired" putrescine oxidase electrode based on unique enzyme combination was presented for the first time. The optimized biosensor had a good selectivity, wide linear measuring range (0.01–0.25 mM), low detection limit (0.005 mM), appropriate operational and storage stability. The putrescine sensor was successfully used for the analysis of beer samples.

Acknowledgments

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