Multienzyme Amperometric Gluconic Acid Biosensor Based on Nanocomposite Planar Electrodes for Analysis in Musts and Wines

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An amperometric biosensor utilizing multi-enzyme cascade consisting of gluconate kinase, creatine kinase, creatinase, sarcosine oxidase and peroxidase for the determination of gluconic acid has been developed. The enzymes were immobilized between chitosan layers onto the surface of planar nanocomposite electrodes containing multi-walled carbon nanotubes. A measuring medium contained adenosine triphosphate, creatine phosphate and hexacyanoferrate(II) as the redox mediator and the current response was measured at constant potential of -50 mV vs. Ag/AgCl reference electrode. The biosensor shows linearity over the range 4 to 620 μM with the detection limit of 2.6 μM, sensitivity of 45.3 nA μM⁻¹ cm⁻² and response time of 70 s, a satisfactory operational stability with no loss of the sensitivity after 30 consecutive measurements and a response ability above 90 % after 3 day of use. Its storage stability is given by about 90% of the initial sensitivity after 12 months (stored at room temperature). The results of the determination of gluconic acid in musts and wines correspond well with those achieved by HPLC as the reference method.

Keywords: gluconic acid; amperometric multi-enzyme biosensor; nanocomposite electrode; wine; must

1. INTRODUCTION

Gluconic acid, an oxidation product of glucose, occurs widely in nature. It is present in various foodstuffs naturally or as a permitted additive [1]. In wine industry, gluconic acid represents the most important indicator of infection of grapes by Botrytis Cinerea [2]. This fungal infection should be avoided since it affects negatively the quality of grapes and, consequently, wines resulting in serious
economic losses of wineries on the worldwide basis. Thus a rapid, specific, simple and cost effective determination of gluconic acid in musts and wines, which could be performed directly in wineries, is highly required [3]. A variety of analytical techniques such as chromatography [4-6], electrophoresis [7], infrared spectroscopy [8], spectrophotometry [9], chemiluminescence [10] and fluorescence [11] have been developed for this purpose. Enzyme based electrochemical biosensors seem to have a potential to fulfil well all of the requirements mentioned above [12-14].

Ikeda et al. [15] constructed amperometric biosensor based on gluconate dehydrogenase (GADH) and p-benzoquinone as an electron-transfer mediator. This concept has been improved by GADH coimmobilization with tetrathiafulvalene (TTF) using self-assembled monolayers on the surface of gold disc [16] and screen-printed carbon electrode (SPCE) [3]. As GADH has been not longer commercially available, alternative systems have been described employing gluconate kinase (GK) and 6-phospho-d-gluconate dehydrogenase (6PGDH) coimmobilized by an entrapment within the polysulfone membrane on a graphite-epoxy composite [13] and SPCE [14]. Both of these biosensors operated at the relatively high working potential of 800 mV with coenzymes ATP and NADP in measuring media. Recently, the detection of NADPH formed by 6PGDH at suitable low potential of 100 mV was described using film of polyaniline/poly(2-acrylamido-2-methyl-1-propanesulfonic acid) electrodeposited on SPCE [17]. Although these biosensors showed a sufficient analytical performance, they exhibited relatively short lifetimes from several days to 3 months. Storage and operational stabilities are crucial factors for the potential biosensor commercialization, together with production costs and the availability of components. To fulfil such a requirement, a glycerol biosensor based on a novel multi-enzyme cascade with an extended lifetime (at least to 15 months) has been reported recently creating a new platform for the biosensor preparation with diverse kinases [18].

Here we present the gluconic acid biosensor utilizing a multi-enzyme cascade consisting of gluconate kinase, creatine kinase, creatinase, sarcosine oxidase and peroxidase immobilized onto nanocomposite planar electrodes containing multi-walled carbon nanotubes. All these enzymes are available, cheap, very stable and cooperating effectively at suitable working conditions allowing thus fabrication of sensitive, robust and stable biosensors. In addition, the use of expensive NADP cofactor is avoided. The aim of this work was the construction of a gluconate biosensor for analyses of real oenological samples and compatible with a commercially available portable analytical device like Omnilab W [19].

2. EXPERIMENTAL

2.1 Reagents and electrode substrate

Sarcosine oxidase, creatinase, horseradish peroxidase were obtained from Sorachim (Lausanne, Switzerland), gluconate kinase from CPC Biotech (Burago di Molgora, Italy) and creatine kinase from USB (Cleveland, OH USA). Potassium hexacyanoferrate(II), N-eicosane, potassium D-gluconate, D-glucose, D-fructose, sodium-L-lactate, L-ascorbic acid, L-malate disodium salt, sodium acetate, anhydrous ethanol, magnesium sulphate heptahydrate, glycerol, adenosine 5’-triphosphate (ATP), creatine phosphate (CP) and chitosan from shrimp shells (85% deacetylated) were supplied by Sigma-
Aldrich (St. Louis, USA). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Riedel-de Haen (Seelze, Germany). All chemicals used were of analytical grade. Nanopure water (Millipore Milli-Q, 18 MΩ cm) was used throughout.

Multi-walled carbon nanotubes (MWCNT) (d = 60-100 nm, l = 5-15 μm, 95 % purity) were purchased from NanoAmor (Houston, USA). Basic planar circular electrodes with the diameter of 1.6 mm and equipped with a Ag/AgCl reference electrode of diameter 2 mm screen-printed on the planar glass-epoxy-laminate substrate were obtained from Biorealis (Bratislava, Slovakia).

2.2 Preparation of nanocomposites and the biosensor

Slightly modified procedure described previously [18, 20] was used for the nanocomposite preparation. First, 100 mg of N-eicosane were melted at the temperature 45°C. Then, 12 mg of MWCNT were added and the mixture was stirred vigorously with a mini-stirrer until the homogenous mixture was obtained. The suspension was subsequently deposited on the surface of cleaned (nanopure water and ethanol) metal contacts to create thick layer circles. Then, the nanocomposite layer was left to solidify [21].

The immobilization of individual enzymes with optimum amounts of 0.25 U of GK, 0.15 U of CK, 0.25 U of SAOX, 0.6 U of CRE and 1 U for HRP dissolved in nanopure water on the electrode surface was carried out by their sandwiching between two chitosan layers formed by applying its 1 % (w/w) solution. Individual layers were deposited after the previous one was dried. The prepared biosensor was stored at room temperature in a desiccator until use.

2.3 Amperometric measurements

Electrochemical measurements were performed with the electrochemical analyzers Autolab M101 (Metrohm Autolab, Netherlands) and Omnilab (Biorealis, Bratislava, Slovakia). Chronoamperometry was performed by applying selected constant potential of -0.5 V vs. Ag/AgCl after inserting the biosensor into a measuring solution either of 1 mL in microtube or 10 mL in beaker under stirring at laboratory temperature. The concentrations of reagents in the working medium of 0.1 M phosphate buffer pH 7.5 were as follows: 7.5 mM magnesium ions, 1 mM CP and 0.5 mM ATP, 2.5 mM hexacyanoferrate(II). The constant working potential for amperometric detection was -50 mV vs. Ag/AgCl. After the measurement, the biosensor was stored in 0.1 M phosphate buffer solution of pH 7.5 at laboratory temperature (up to 10 hours). For the storage stability studies, the biosensor was kept dry in a desiccator at room temperature or at 4 °C (for long term stability studies).

2.4 HPLC analysis

HPLC determination of organic acids was performed on a DeltaChromTM liquid chromatograph (Watrex, Bratislava, Slovakia) equipped with Applied Biosystems 759A absorbance detector (210 nm) (San Diego, CA, USA). Experimental conditions were: stationary phase - column
Polymer IEX in H$^+$ form, 250 mm x 8 mm, 8 μm in diameter (Watrex, Bratislava, Slovakia), guard column Polymer IEX in H$^+$ form, 10 mm x 4 mm, 8 μm (Watrex, Bratislava, Slovakia); column temperature 50 °C; mobile phase with isocratic elution - 9 mM H$_2$SO$_4$ in water for HPLC; flow rate 1.0 mL min$^{-1}$. Data were collected and processed by the software Clarity chromatography station DataApex (Prague, Czech Republic). Prior to analysis, samples were diluted by a mobile phase and filtered through 0.22 μm Chromafil AO filters (Macherey-Nagel, Düren, Germany). Organic acids were identified by a comparison with retention times of the authentic standard solutions.

3. RESULTS AND DISCUSSION

Figure 1. Scheme of multi-enzyme cascade immobilized between chitosan layers at the gluconic acid biosensor with hexacyanoferrate(II) as the redox mediator in solution. The Med$_{ox}$ reduction current is measured at the applied constant potential of -50 mV vs. Ag/AgCl.

The reaction scheme of multi-enzyme cascade is shown in Figure 1. The hexacyanoferrate(III) ion formed as a product of the cascade reaction is reduced to hexacyanoferrate(II) at the electrode using an applied constant potential where the current is proportional to the gluconic acid concentration. A closed contact of the immobilized enzymes leads to their cooperation which results in rapid and sensitive sensor response.

3.1 Optimization of working conditions

The quantities of immobilized enzymes were optimized in the range 0.1 to 0.8 U for gluconate kinase (GK), 0.1 to 0.5 U for creatine kinase (CK) and sarcosine oxidase (SAOX), 0.3 to 1.5 U for creatinase (CRE) and 0.5 to 4 U for horseradish peroxidase (HRP). High enzyme loadings led to the decrease in current which is likely due to the steric hindrance of protein immobilized on the electrode surface. On the other hand, low enzyme quantities caused a decrease of the biosensor response and narrow linear concentration ranges. The optimum amounts found were 0.25 U of GK, 0.15 U of CK, 0.25 U of SAOX, 0.6 U of CRE and 1 U for HRP and, consequently, used in further experiments.
Concentration of the mediator in the working medium was studied in the range from 1 to 10 mM hexacyanoferrate(II) where 2.5 mM was found as the optimum. The concentrations of magnesium ions (typical kinase activator) and cofactors CP and ATP were optimal at 7.5 mM, 1 mM and 0.5 mM, respectively. Recently similar concentrations (Mg$^{2+}$ 7.0 and 2.4 mM, ATP 0.36 and 0.39 mM) were published in two reports on the bienzymatic gluconic acid biosensors [14, 17].

A selection of the suitable working potential plays an important role in a suppression of possible interferences such as wine polyphenols. A direct detection of hydrogen peroxide (Figure 1) could be also utilized at the potential over 600 mV which is accompanied by a risk of false interfering signals. For this reason, peroxidase with the hexacyanoferrate(III/II) redox couple were employed allowing the detection at a significantly lower potential. A working potential value was tested within the range -200 mV to +150 mV vs Ag/AgCl and that of -50 mV has been selected as possessing satisfactory biosensor sensitivity and avoiding from undesired interferences. This was proved in next experiments.

![Figure 2](image_url)

**Figure 2.** Effect of pH on the gluconic acid biosensor relative response (pH 7.5 correspond to 100%). Experimental conditions: 5 mM hexacyanoferrate(II), 50 μM gluconic acid, applied constant potential -50 mV vs. Ag/AgCl.

As optimum pH for single used enzymes varies, selection of suitable pH value for multienzyme biosensor was crucial for its good functionality and stability. The dependence of the amperometric response of the biosensor after addition of 10 mM gluconic acid (pK$_a$ 3.86) on pH of the phosphate buffer solution (PB) is shown in Fig. 2. The current value increased with increasing pH until and reached the maximum at the pH 7.5. Stability of the biosensor was optimal at this pH as well.
3.2 Analytical characteristics

Amperometric response of the biosensor to increasing concentration of gluconic acid at optimized conditions is plotted in Figure 3.

![Figure 3. Calibration curve obtained for the gluconic acid biosensor. Experimental conditions: 5 mM hexacyanoferrate(II), 0.1 M phosphate buffer, pH 7.5, applied constant potential -50 mV vs. Ag/AgCl.](image)

The biosensor exhibited a good linearity within the range 4-620 µM with the correlation coefficient of 0.990 (n = 21). The detection limit obtained was 2.6 µM (based on signal/noise = 3) and the sensitivity 45.3 nA µM⁻¹ cm². These characteristics are better or comparable to those reported previously for diverse gluconate biosensors with exception of the significantly lower detection limit 0.19 µM [16]. The response of the presented biosensor was relatively fast. The current started to increase approximately 4 s after the injection of standard or sample solutions reaching a 90% steady-state current within 45 s. The final response time was found to be about 70 s. Reproducibility of the measurement with the same biosensor was tested as a current response on 50 µM gluconic acid and at the average response of 42.7 nA is represented by RSD of 4.17% (n=6). In Table 1, analytical parameters of the biosensor developed in this work are compared with those reported previously.

3.3 Stability study

Operation and storage stabilities of the prepared biosensor were tested. No loss of the biosensor sensitivity after 30 successive additions of 50 µM gluconic acid or wine samples was observed. The biosensor exhibited a response above 90 % or above 55 % of the original one after 3 days or 7 days, respectively, at storage in 0.1 M PB of pH 7.5 at 4 °C after a periodic use. Humidity and high
temperature are typically considered as the most negative factors which can affect the storage stability of enzymatic biosensors. To perform the long term stability study, a batch of 80 biosensors was prepared. The sensitivities of 5 biosensors from this batch were evaluated in regular intervals. When the biosensors were held in a desiccator at room temperature without any previous use for 12 months, their sensitivity remained about 90 % of the initial value. We suppose that the long storage and operation stability was achieved due to good stability of the immobilization of employed enzymes in the chitosan matrix. Such excellent long-term storage stability was observed in our laboratory also at other biosensors based on the chitosan sandwich as it was discussed in the previous work [22]. With respect to facts that the stability contributes to a reliability of biosensors and could significantly reduce the operational costs of potential users, this is of great importance for the expected use of the biosensor in control processes and routine monitoring. The stability of the present biosensor is significantly higher when compared to those reported in the literature (Table 1).

**Table 1.** Comparison of analytical parameters obtained with the electrochemical gluconic acid biosensors.

<table>
<thead>
<tr>
<th>Biosensor composition</th>
<th>Concentration range (μM)</th>
<th>LOD (μM)</th>
<th>Stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADH/screen-printed carbon electrode</td>
<td>9.0 - 131.4</td>
<td>4.2</td>
<td>15 days</td>
<td>3</td>
</tr>
<tr>
<td>GK+6PGDH/graphite epoxide composite electrode</td>
<td>7.0 - 250</td>
<td>7.0</td>
<td>5-7 calibrations</td>
<td>13</td>
</tr>
<tr>
<td>GK+6PGDH/screen-printed carbon electrode</td>
<td>7.46 - 71.43</td>
<td>7.46</td>
<td>not given</td>
<td>14</td>
</tr>
<tr>
<td>GADH/carbon paste electrode</td>
<td>100 - 4000</td>
<td>not shown</td>
<td>1 month</td>
<td>15</td>
</tr>
<tr>
<td>GADH with TTF/gold electrode</td>
<td>0.6 - 20</td>
<td>0.19</td>
<td>53 days</td>
<td>16</td>
</tr>
<tr>
<td>GK+6PGDH/screen-printed carbon electrode</td>
<td>5 - 100</td>
<td>3</td>
<td>90 days</td>
<td>17</td>
</tr>
<tr>
<td>GK+CK+CRE+SAOX+HRP/MWCNT nanocomposite electrode</td>
<td>4 - 620</td>
<td>2.6</td>
<td>12 months</td>
<td>this work</td>
</tr>
</tbody>
</table>

### 3.4 Interferences

Generally the interferences are one of the more important factors affecting a relatively low dissemination of biosensors in various sectors. The interference studies were performed to verify a specificity of the proposed biosensor to the gluconate determination. Various components of wine may affect the accuracy of analysis results by interfering with enzymes activity or by direct oxidation on the electrode. Particularly, polyphenols, alcohols, sugars and some organic acids should be considered as possible interferences. The effect of potential interferences present in musts and wines such as ethanol (10%), glucose (18 g L\(^{-1}\)), fructose (18 g L\(^{-1}\)), glycerol (10 g L\(^{-1}\)), L-lactic (4 g L\(^{-1}\)), citric (2 g L\(^{-1}\)), tartaric (4 g L\(^{-1}\)), malic (4 g L\(^{-1}\)), acetic (2 g L\(^{-1}\)) and L-ascorbic acid (50 mg L\(^{-1}\)) on the response of the biosensor was evaluated. The presence of sugars, ethanol and acids did not change the current signal. The effect of polyphenols was also tested by the addition of red wines and using bare electrodes covered with chitosan double layer without enzymes and the optimized composition of measuring
solution. No significant current changes in the biosensor response were observed, thus, polyphenols also do not interfere under the proposed experimental conditions.

3.5 Real samples analysis

Finally, the presented multi-enzyme biosensor was applied to the analysis of gluconic acid in various musts and wines without any pretreatment. The results obtained are summarized in Table 2.

The determination was performed by injecting 10 μL of sample and calibration solution in 1 mL of measuring media. As it can be seen in Table 1 that the results obtained with the biosensor are in good agreement with those obtained by HPLC as the reference method. The analyzed musts and wines contained low amounts of gluconic acid apart from Tokaj. So the samples were spiked with a known concentration of gluconic acid. Recovery values about 100 % demonstrate a good reliability of the proposed biosensor. Tokaj is a noble Slovak wine naturally containing gluconic acid as a result of particular cultivation conditions and production process.

Table 2. Results of the determination of gluconic acid in must and wine samples using the biosensor and HPLC as a standard analytical method. Results are expressed with standard deviation (n=3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gluconic acid added (g/l)</th>
<th>Gluconic acid found (g/l)</th>
<th>Recovery (%)</th>
<th>Gluconic acid determined by HPLC (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White must</td>
<td>-</td>
<td>0.04± 0.01</td>
<td>-</td>
<td>0.031± 0.007</td>
</tr>
<tr>
<td>White must</td>
<td>0.5</td>
<td>0.51± 0.03</td>
<td>94.4</td>
<td>0.545± 0.022</td>
</tr>
<tr>
<td>Red must</td>
<td>-</td>
<td>0.11± 0.02</td>
<td>-</td>
<td>0.092± 0.011</td>
</tr>
<tr>
<td>Red must</td>
<td>1.0</td>
<td>1.16± 0.06</td>
<td>104.5</td>
<td>1.097± 0.039</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>-</td>
<td>0.03± 0.01</td>
<td>-</td>
<td>0.012± 0.003</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>0.5</td>
<td>0.49± 0.04</td>
<td>92.5</td>
<td>0.526± 0.031</td>
</tr>
<tr>
<td>Pinot white</td>
<td>-</td>
<td>0.00± 0.00</td>
<td>-</td>
<td>0.011± 0.013</td>
</tr>
<tr>
<td>Pinot white</td>
<td>1.0</td>
<td>0.96± 0.07</td>
<td>96.0</td>
<td>1.003± 0.042</td>
</tr>
<tr>
<td>Tempranilo</td>
<td>-</td>
<td>0.12± 0.02</td>
<td>-</td>
<td>0.149± 0.015</td>
</tr>
<tr>
<td>Tempranilo</td>
<td>0.5</td>
<td>0.59± 0.04</td>
<td>103.5</td>
<td>0.633± 0.030</td>
</tr>
<tr>
<td>Alibernet</td>
<td>-</td>
<td>0.07± 0.01</td>
<td>-</td>
<td>0.048± 0.010</td>
</tr>
<tr>
<td>Alibernet</td>
<td>1.0</td>
<td>1.12± 0.04</td>
<td>104.7</td>
<td>1.104± 0.047</td>
</tr>
<tr>
<td>Tokaj</td>
<td>-</td>
<td>0.46± 0.03</td>
<td>-</td>
<td>0.491± 0.021</td>
</tr>
</tbody>
</table>
4. CONCLUSIONS

The novel multienzyme biosensor based on commercially available materials for the selectively quantification of gluconic acid is reported. 5 enzymes were immobilized between chitosan layers on the surface of planar nanocomposite electrode. The simple and effective immobilization technique provided long-term storage stability, low fabrication costs, and good analytical performance. The biosensor exhibited wide linear range, low detection limit, high sensitivity, short measuring time and interference-free measurements. It was successfully applied to gluconic acid determination in real must and wine samples, validating the biosensor performance through comparison with the reference HPLC method. The performance characteristics of this new analytical tool may satisfy requirements of wine industry being particularly suitable for small-medium winemakers. The developed biosensor is compatible with the small device Omnilab W.

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