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Application of immobilized citrate lyase in a bioanalytical system for determination of citrates

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A bioanalytical protocol based on the enzymatic-spectrophotometric micro-method for determination of citrate with immobilized citrate lyase (CL) is described. In the design of this spectrophotometric bioanalytical system, L-malate dehydrogenase (L-MDH), oxaloacetate decarboxylase (OACD) and L-lactate dehydrogenase (L-LDH) were applied as a biological recognition system. Immobilized CL catalysed the conversion of citrate to oxaloacetate and acetate. In the presence of the enzymes L-MDH and L-LDH, oxaloacetate and its decarboxylation product pyruvate were reduced to L-malate and L-lactate, respectively. The concentration of citrate is stoichiometric to the concentration of nicotinamide adenine dinucleotide (NADH) formed in enzymatic reaction. NADH was determined by means of its light absorbance at 340 nm. The maximal difference in light absorbance was detected when CL had been immobilized on porous carbon. If CL had been immobilized on wool fibres or carbon rod the absorbance difference after 360 min was almost 2 times and 3.2 times lower, respectively, as compared with the results observed when CL had been immobilized on porous carbon. The method proposed here has several advantages if compared with the technique based on dissolved CL. Moreover, this method allows achieving a higher rate of CL-catalysed reaction and to reduce the duration of analysis versus the previously suggested methods. We expect that this method can be employed for determination of other analytes by applying the corresponding enzymes in the design of the bioanalytical system.

Key words: bioanalytical chemistry, enzymatic detection, biosensors, citrate lyase, citric acid, citrate

INTRODUCTION

An important field of analytical chemistry is determination of biologically active substrates. Here, the most promising are bioanalytical systems that are based on immobilized enzymes [1] or some affinity exhibiting materials [2] because such systems allow to determine a variety of biologically active analytes (e. g., glucose, creatine, citric acid, citrates and many others) [3]. Citric acid and citrates are present in numerous natural products. They are very important intermediates in the metabolism of plants, animals and microorganisms. In the human body, citrates are metabolised and excreted by the kidney [4]. Citrates are products of normal oxidative pathways in the body of mammalians and are normally excreted in the urine [5]. Urinary citrates play an important role in the endogenous inhibiting of the formation of calcium-containing kidney stones [6]. Citrates form stable soluble complexes by chelating calcium [7], directly preventing the crystallization and precipitation of calcium-containing compounds [8]. Moreover, citrates interact with Tamm–Horsfall proteins and inhibit Ca-oxalate crystallization [9]. Low urinary citrate concentrations are found in approximately half of adult patients with renal stones [10]. Citric acid is used in the food and beverage industry to improve and protect flavour and aroma, and in cosmetics for pH adjustment. It is a component of many pharmaceuticals and washing powders.

Determination of citric acid and citrates has become an important item for biological fluids (e. g., serum, urine, plasma, seminal, sperm plasma) and foodstuffs. Several methods have been proposed for the determination of citrates based on ion-exchange chromatography [11], high performance liquid chromatography (HPLC) [12], capillary electrophoresis [13] and isotachophoresis [14]. These methods are time-consuming procedures; besides, sample preparation is required in order to separate citric acid from other co-existing tricarboxylic acids. Other approaches based on conductometric or spectrophotometric methods [15] suffer from selectivity as they are based on non-specific reactions with carboxylic acids. The above-mentioned selectivity problems could be potentially solved by using a high specificity enzyme such as citrate lyase (CL) [16–19]. Enzymatic methods allow a rapid and reliable determination of several constituents. Enzymes are valuable analytical tools and offer sensitive and specific methods for quantitative analysis; especially useful and reliable are bioanalytical systems based on immobilized enzymes [20]. Firstly, the procedure utilizing

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soluble citrate lyase was reported by Stern as far back as 1955 [21]. The procedure proposed by Dagley [22] was based on the fact that the oxaloacetate formed from citrate is decomposed to pyruvate by oxaloacetate decarboxylase present in cell-free extracts. Moellering and Gruber [23] reported a method with soluble CL, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), where a decrease in the absorbance of the NADH at 340 nm was monitored. Since then, several other approaches have been proposed: (i) some of them using soluble CL and immobilized MDH with NADH [24] detection; (ii) others applying CL and oxaloacetate decarboxylase (OACD) in soluble [25] or immobilized [26] form in conjunction with polarography; (iii) in the next class of bioanalytical systems, direct amperometric determination of citric acid has been proposed [27] in connection with ascorbate oxidase to eliminate ascorbic acid interference. Gajovic et al. [28] used CL entrapped in gelatine.

The aim of this study was to investigate the possibility to apply immobilized citrate lyase for enzymatic determination of citrates.

EXPERIMENTAL

Chemicals. Citrate lyase (EC 4.1.3.6). The enzyme had an activity of 12 U/mg. Lyophilisate, consisting of glycylglycine buffer, pH 7.8, L-malate dehydrogenase (EC 1.1.1.37, 136 U/mg), L-lactate dehydrogenase (EC 1.1.1.27, 280 U/mg), oxaloacetate decarboxylase (EC.4.1.1.3, 47 U/mg). NADH and stabilizers. Citric acid standard solution (200 mM). All chemicals were purchased from Boehringer Mannheim (Mannheim, Germany).

Immobilization of CL. For immobilization of CL, 200 μl of 20 mg/ml CL solution was distributed on the surface of carriers: porous carbon, a carbon rod and wool fibres. Between the measurements, the carriers with immobilized CL were stored in a refrigerator at +4 °C. Before investigations, all carriers with immobilized CL (Figure) underwent an optimal pre-treatment period for 5 min in a glycylglycine buffer at pH 7.8 (data not shown). This pre-treatment was performed in order to remove weakly adsorbed CL from the surface of carriers.

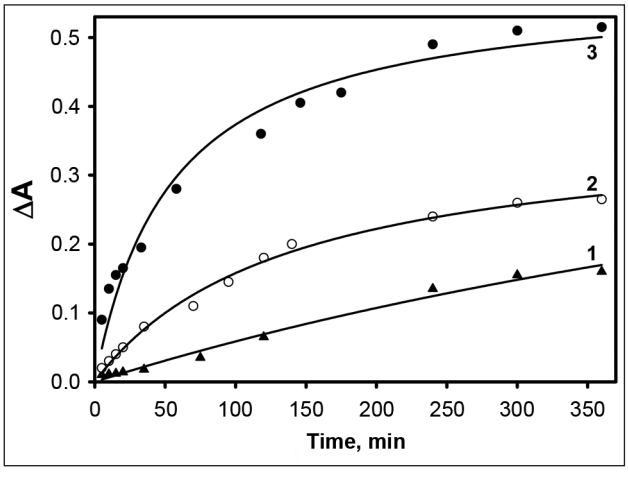

Figure. Changes in optical absorbance at 340 nm *vs.* incubation period. Carbon rod (1); wool fibres (2) and porous carbon (3) with immobilized CL were immersed in 2 ml glass cuvettes containing control and sample solutions for a period indicated on the X-axis.

Porous carbon (Reticulated Vitreous Carbon foam (pores/cm: 24, porosity: 96.5%, specific surface area: 3.78 $mm^2\ mm^{-3}$, bulk density: 0.05 gcm^{-3}) was purchased from Goodfellow Cambridge Ltd. (Cambridgeshire, UK). Porous carbon was cut into 0.5 cm^3 tablets which were used for CL immobilization.

Carbon rods (diameter: 3 mm) of Ultra F purity were obtained from Ultra Carbon Division of Carbon (RAVEN-M, USA). For CL immobilization, 2 cm long carbon rods were used.

Sheep wool fibres were used in this study. Wool fibers were cleaned from the native covalently bound surface lipid layer by an acetone / ethanol mixture for 12 h; then they were rinsed with distilled water and dried at room temperature to achieve the constant weight. For CL immobilization, 0.3 × 2 cm pieces of wool fibre were used.

To estimate the efficiency of the immobilization procedure, samples with adsorbed CL were incubated in glycylglycine buffer, pH 7.8, for 5 min under mixing. Then the samples were removed from the reaction vessel, and citric acid was added to this solution. The percentage of the desorbed enzyme was estimated by calculating the activity of CL in glycylglycine buffer, pH 7.8, which was used for adsorption of weakly immobilized CL.

Spectrophotometrical measurements. Photometrical measurements were performed with a Perkin-Elmer Lambda 25 spectrophotometer (Friedrichshafen, Germany).

The activity of immobilized CL was determined by a spectrophotometric test. The control and sample solutions were prepared from the lyophilisate which consisted of glycylglycine buffer, L-malate dehydrogenase, L-lactate dehydrogenase, NADH and stabilizers (0.1167 g/ml) dissolved in bidistilled water. Standard solution of citric acid was added only to the sample solutions; the final concentration of citric acid in the sample solution was 12 mM. Porous carbon, the carbon rod and wool fibres with immobilized CL were immersed in 2 ml volume glass cuvettes containing control and sample solutions, respectively (l = 1 cm, 1.5 ml volume of solution was used, at 20–25 °C) for a period presented in Figure. During the spectrophotometric measurements, porous carbon, the carbon rod or wool fibres with immobilized CL were removed from the spectrophotometer cuvettes.

Measurements of enzyme-immobilization efficiency were performed consecutively in two samples containing 12 mM of citrate. The efficiency of immobilization was calculated according to the formula $E_i = A_{(2)} \cdot 100/A_{(1)}$ (%), where $A_{(1)}$ is activity of enzyme detected in the first measurement immediately after the immobilization procedure; $A_{(2)}$ is activity of enzyme detected in the second measurement. The relative amount of enzyme (L), which leached from the immobilization substrate, was calculated according to the formula $L = 100 - E_i$ (%), where E_i is the efficiency of enzyme immobilization.

Sensitivity of the method was determined by comparing analytical signals with the natural noise of the analytical system. To this end, an incubation of CL-modified substrates in different concentrations of citrate was performed for 5 min. After a 5 min of incubation period, spectrophotometrical measurements which are described previously were done, and then the obtained analytical signals were compared with the level of natural noise of the analytical system.

Calculation of the coefficient of variation (C_V) was calculated from 12 consecutive measurements performed with each of

CL-modified substrates. The citrate concentration in C_V measurements was 12 mM, and the incubation period was 5 min. The coefficient of variation was calculated according to the formula: $C_V = \sigma \cdot 100/\mu$ (%); where σ is standard deviation and μ is the mean value of the analytical signal calculated from all 12 mesurements.

Immobilized CL catalyses the conversion of citric acid (citrate) to oxaloacetate and acetate (1):

$$\text{Citrate} \xrightarrow{\textbf{CL}} \text{oxaloacetate} + \text{acetate}. \quad (1)$$

In the presence of the enzymes L-malate dehydrogenase (L-MDH), oxaloacetate decarboxylase (OACD) and L-lactate dehydrogenase (L-LDH), oxalacetate (2) and its decarboxylation product pyruvate (3) are reduced to L-malate (3) and L-lactate (4), respectively, by reduced nicotinamide adenine dinucleotide:

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\textbf{L-MDH}} \text{L-malate} + \text{NAD}^+, \quad (2)$$

$$\text{Oxaloacetate} \xrightarrow{\textbf{OACD}} \text{pyruvate} + \text{CO}_2, \quad (3)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\textbf{L-LDH}} \text{L-lactate} + \text{NAD}^+. \quad (4)$$

The addition of L-LDH ensures that any oxalacetate converted into pyruvate is measured. This conversion to pyruvate occurs by either OACD, which may be present in CL [23], or by the nonenzymatic reaction as described by Moellering and Gruber [29]. The concentration of NADH oxidized in reactions (2) and (3) is proportional to the concentration of citrate. The absorption of NADH was measured at 340 nm (ε = 6.3 l/mmol · cm). The differences in absorbance between control and sample solutions were determined by the equation:

$$\Delta A = A_{\textit{sample solution}} - A_{\textit{control solution}}. \quad (5)$$

RESULTS AND DISCUSSION

Immobilized CL catalysed the conversion of citric acid into oxaloacetate and acetate, thus enabling in this reaction oxidized NADH to be spectrophotometrically registered. The control experiments, performed in order to check the efficiency of immobilization, showed that after the first 5 min of treatment with buffer solution all carriers (porous carbon, the carbon rod and wool fibres) released a significant amount of the adsorbed enzyme. Measurements of enzyme-immobilization efficiency were performed consecutively in two samples of citrate: the first measurement was performed immediately after the immobilization procedure and was compared with the second measurement performed consecutively in a sample containing the same concentration of citrate. The calculated relative amount of leached CL was equal to 18–21% of CL used for the immobilization procedure and in this amount was independent on the nature of the carrier studied. This experiment proves the fact that a weakly adsorbed enzyme was by this treatment desorbed from the surface of the study material. During the second and third treatments for 5 min in buffer solution, porous carbon released less than 1% of CL, however, adsorption on carbon the rod and wool fibres was weaker, and more than 2% of CL was released into solution. The presented data prove that citrate lyase is successfully immobilized on different surfaces – porous carbon, carbon rod and wool fibres. Standard variation in the case when CL was immobilized on the carbon rod was 10 (%); on the wool fibres it was 8 (%), and if CL was immobilized on porous carbon, the C_V was in the range of 4.5 (%). The calculations of C_V demonstrates the best applicability of porous carbon for developing a bioanalytical system for citrate determination.

The maximal absorbance difference between the control and sample reactions was detected when CL was immobilized on porous carbon (Figure, curve 3). In case of CL immobilized on wool fibres (Figure, curve 2), the absorbance after 360 min was almost two times less, and in the case of CL immobilized on carbon rod (Figure, curve 1) almost 3.2 times less than that measured for porous carbon.

The sensitivity of the method was determined by comparing the analytical signals with the natural noise of the analytical system, and it was detected that for measurements based on the application of carbon rods modified with CL it was 5 ± 1 mM; in the case of wool fibres it was 2.2 ± 0.6 mM and in the case of porous carbon 0.8 ± 0.3 mM.

A rapid, convenient enzymatic-spectrophotometric micromethod for the determination of citrate with immobilized citrate lyase (CL) on different substrates is proposed. In this system, CL is immobilized on porous carbon, carbon rod or wool fibres. Other enzymes – L-MDH and L-LDH – are in a soluble form. A highly porous and conducting carbon electrode is used for physical adsorption of glucose oxidase and lactate oxidase. The porous carbon provides a suitable matrix for a simple and reproducible construction of the sensor [30]. The diffusion and adsorption of serine proteases on wool fibres were analysed and found to be dependent on the size of the protease [31].

The method proposed here has several advantages if compared with the spectrophotometrical technique utilizing soluble citrate lyase, proposed by Stern [21]. No addition of magnesium ions was required, whereas Stern's method utilizes magnesium ions and suffers from a limitation: a given amount of citrate lyase catalyses the decomposition of only a limited amount of citrate before the enzyme becomes inactive. The method presented here has an important advantage as compared with the one presented by Moellering and Gruber [23] and some other methods based on soluble enzymes [24, 25] since the immobilization of CL reduces its consumption. In the enzymatic-spectrophotometric method proposed here, immobilized CL catalysed the conversion of citrate to oxaloacetate and acetate in a highly specific way under physiological conditions without influencing the balance of the reaction. In the presence of the soluble enzymes, oxalacetate and its decarboxylation product pyruvate were reduced to L-malate and L-lactate; this reaction was accompanied by NADH oxidation. The concentration of citrate was stoichiometric to the concentration of nicotinamide adenine dinucleotide (NADH) oxidized in these reactions [17, 23, 32, 33]. The best results were obtained when CL was immobilized on a porous carbon substrate. This substrate has a larger surface area compared to carbon rod. An equal amount of enzyme, when distributed on the higher surface area, exhibits a higher activity. A lower activity of the used enzyme

when immobilized on hydrophobic material can be explained by its hydrophobic nature. The wool fibres are hydrophobic, and hydrophilic enzymes (e. g., glucose oxidase (GOx) [34]) are weakly adsorbed on wool fibres [35].

Furthermore, the method presented here is comparable with the methods where the same enzyme is entrapped within gelatine, as proposed by [28].

CONCLUSIONS

This preliminary study shows that CL immobilized on porous carbon together with soluble L-MDH, OACD and L-LDH can be applied in the design of a spectrophotometric bioanalytical system for a rapid determination of citrates.

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IMOBILIZUOTOS CITRAT-LIAZĖS TAIKYMAS BIOANALIZINĖJE SISTEMOJE, SKIRTOJE CITRATAMS NUSTATYTI

S a n t r a u k a

Sukurtas ir aprašytas fermentinis-spektrometrinis mikrometodas nustatyti citratams, panaudojant imobilizuotą citrat-liazę (CL). Spektrofotometrinei analitinei sistemai kurti taip pat buvo panaudoti neimobilizuoti fermentai L-malato dehidrogenazė (L-MDH), oksalo acetato dekarboksilazė (OLCH) ir L-laktato dehidrogenazė (L-LDH). Šiame darbe CL buvo imobilizuota ant porėtos anglies, anglinio strypelio ir vilnos. Imobilizuota CL katalizavo citrato virtimą oksalo acetatu ir acetatu. Tirpale esant fermentams L-MDH ir L-LDH oksalo acetatas ir jo dekarboksilinimo produktas piruvatas buvo redukuojami atitinkamai iki L-malato ir L-laktato. Citrato koncentracija yra stechiometrinė nikotinamido adenino dinukleotido (NADH) koncentracijai. NADH koncentracija buvo nustatoma pagal jo optinio tankio reikšmę esant 340 nm bangos ilgiui. Maksimalus optinio tankio skirtumas buvo nustatytas CL imobilizavus ant porėtos anglies. Imobilizavus CL ant vilnos ir anglinio strypelio optinio tankio skirtumas po 360 minučių buvo mažesnis atitinkamai beveik 2 ir 3,2 karto, lyginant su CL imobilizuota ant porėtos anglies. Mūsų pasiūlytas citratų nustatymo metodas, lyginant su metodais, kuriuos taikant naudojama tirpi CL, įgalina panaudoti šį fermentą daugeliui tyrimų ir sumažina bendrą analizės trukmę. Tyrimų rezultatai leidžia tikėtis, kad ateityje šioje publikacijoje aprašytais principais pagrįstą analizinę sistemą būtų galima taikyti citratų koncentracijai maisto produktuose bei biologiniuose tirpaluose nustatyti.