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# Biosensor for rapid determination of 3-hydroxybutyrate using bienzyme system

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**Abstract**

A bienzyme-based Clark electrode was developed for the determination of 3-hydroxybutyrate. This sensor is based on the specific dehydrogenation by 3-hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30) in combination with salicylate hydroxylase (SHL E.C. 1.14.13.1). The enzymes were entrapped by a poly(carbamoyl) sulfonate (PCS) hydrogel on a Teflon membrane. The principle of the determination scheme is as follows: the specific detecting enzyme, HBDH, catalyses the specific dehydrogenation of 3-hydroxybutyrate consuming $NAD^+$. The products, NADH, initiate the irreversible decarboxylation and the hydroxylation of salicylate by SHL in the presence of oxygen. SHL forces the equilibrium of dehydrogenation of 3-hydroxybutyrate by HBDH to the product side by consuming NADH. Dissolved oxygen acts as an essential material for SHL during its enzymatic reactions. This results in a detectable signal due to the SHL-enzymatic consumptions of dissolved oxygen in the measurement of 3-hydroxybutyrate. Interferences from different amino acids and electroactive substances were found to be minimal due to the specificity of HBDH and the application of a Teflon membrane. The sensor has a fast response (2 s) and short recovery time (2 min) with a linear range between 8 and 800 μM 3-hydroxybutyrate and a detection limit of 3.9 μM. A good agreement ($R^2 = 0.9925$) with theoretical calculation was obtained in spiked serum sample measurements.


*Keywords:* Biosensor; 3-Hydroxybutyrate; Salicylate hydroxylase; 3-Hydroxybutyrate dehydrogenase

## 1. Introduction

The rapid determination of blood 3-hydroxybutyrate (HB) is important for the clinical diagnosis and management of metabolism disorders of carbohydrates (Kiba et al., 2003). It is an important marker for glycemic control in diabetes mellitus. Normal levels of 3-hydroxybutyrate are recognized below 1 mM while hyperketonemia is defined in the range from 1.1 to 3 mM, and ketoacidosis is defined above 3 mM (Laffel, 1999). Futhermore, a clinical significance, shown as ketone body ratio (acetoacetate/3-hydroxybutyrate), has been found between the concentration of 3-hydroxybutyrate and the pathophysiological state of the liver (Uno et al., 1987). In animal studies, the concentration of 3-hydroxybutyrate reflects the balance between fat mobilization and the animal's capacity to utilize ketone bodies produced (Palleschi et al., 1988; Pires et al., 2003). It has become an index for showing the health and nutritional status of the animal. Therefore, the monitoring of 3-hydroxybutyrate is interesting for clinical fields and agricultural management (Lean et al., 1991; Kiba et al., 1994).

Various methods have been developed for the estimation of 3-hydroxybutyrate concentration in different serum

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and plasma samples. 3-Hydroxybutyrate has been determined by enzymatic dehydrogenation to acetoacetate by 3-hydroxybutyrate dehydrogenase (HBDH), followed by the detection of the reduced form of nicotineamide adenine dinucleotide (NADH) (Williamson and Mellamby, 1974; Brashear and Cook, 1983; Saito et al., 1990). Based on this principle, a flow injection system (FIA) has been constructed for 3-hydroxybutyrate analysis in the fields of sports medicine and animal studies (Pires et al., 2003). Moreover, HBDH coupled NADH oxidase quantified 3-hydroxybutyrate by reducing $NAD^+$ to NADH and subsequent oxidation of NADH with generation of $H_2O_2$. This method was based on the measurement of chemiluminesence formed by the luminol-hexa-cyanoferrate mixture and $H_2O_2$ (Tabata and Totani, 1995). Besides, radioisotopic method, using 2-[U-$^{14}$C]ketoglutarate, HBDH and glutamate dehydrogenase (GlDH) has been reported for the measurement of 3-hydroxybutyrate (Sener and Malaisse, 1990; Ramirez et al., 1991). Furthermore, gas-chromatography (GC) (Kimura et al., 1985) and ion-exchange chromatography (IC) (Kiba et al., 1997) have been coupled with trienzyme systems of HBDH/lactate dehydrogenase (LDH)/acetoacetate decarboxylase (AADC) and HBDH/NADH oxidase/pyranose oxidase, respectively, for the determination of 3-hydroxybutyrate. However, these conventional methods for 3-hydroxybutyrate determinations are tedious and time consuming. The chromatography requires complicated preparation of the enzyme columns and expensive instrumentation. The isotopic method has the disposal problems associated with radioactive waste (Tabata and Totani, 1995). Spectrophotometric monitoring of NADH is interfered by other photometric active substances or suspended particles in the sample. Besides, large amounts of biochemicals are necessary for running FIA system in which enzymes are not immobilized for reuse (Weicker et al., 1984).

The importance of enzyme-based, amperometric biosensors has increased considerably during the past decade due to high selectivity of the biorecognition element and the sensitivity of electrochemical signal transduction. This results in the development of rapid, accurate and easy devices for specific measurement of target analyte in complex matrices such as blood, food product and environmental sample. However, only a few biosensors have been proposed for rapid 3-hydroxybutyrate determinations. Recently, Forrow et al. (2005) has constructed an amperometric electrode for the determination of the ketone body 3-hydroxybutyrate. The electrode incorporated HBDH and is based on the mediated detection of NADH, which was generated after oxidation of 3-hydroxybutyrate. However, this biosensor suffered from loss of enzyme activity due to inhibition of the mediator (1,10-phenanthroline-5,6-dione) and also the interference by electroactive substances.

Recently, the coimmobilization of $NAD(P)^+$-dependent dehydrogenases with salicylate hydroxylase (SHL, E.C. 1.14.13.1) in front of a Clark electrode has been investigated for developing a general type of dehydrogenase-based biosensors (Gajovic et al., 1998; Mak et al., 2003; Kwan et al., 2004). The sensitivity was improved by SHL which recycles the cofactor $NAD^+$ from NADH effectively.

In this paper, amperometric determination of 3-hydroxybutyrate with a bienzyme system is described. The 3-hydroxybutyrate sensor was constructed by immobilizing 3-hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30) and salicylate hydroxylase (SHL, E.C. 1.14.13.1) on a Clark-type oxygen electrode. HBDH catalyses the specific dehydrogenation of 3-hydroxybutyrate Eq. (1).

The product, NADH, starts the following reaction: decarboxylation and the hydroxylation of salicylate by SHL Eq. (2)

$$\text{3-hydroxybutyrate} + NAD^+ \xrightarrow{\text{HBDH}} \text{acetoacetate} + \text{NADH} \quad (1)$$

$$\text{salicylate} + \text{NADH} + O_2 \xrightarrow{\text{SHL}} \text{catechol} + NAD^+ + CO_2. \quad (2)$$

The SHL keeps the equilibrium of dehydrogenation of 3-hydroxybutyrate to the product side by consuming the NADH generated by HBDH. Dissolved oxygen acting as an essential material for the enzymatic activity of SHL is consumed proportionally to the concentration of 3-hydroxybutyrate during the measurements. A detectable signal, caused by the consumptions of the dissolved oxygen by SHL, was monitored at −600 mV versus Ag/AgCl by the Clark electrode. Both enzymes were entrapped by a poly(carbamoyl) sulfonate (PCS) hydrogel, which was sandwiched between a dialysis membrane and a Teflon membrane. Electroactive interferences were eliminated by the Teflon membrane.

According to physiological concentrations, twelve serum samples spiked with various amounts of 3-hydroxybutyrate have been applied to study the sensor performance, whose results were compared with the theoretical values.

## 2. Experimental

### 2.1. Apparatus

A potentiostat (Biometria EP 30, Göttingen, Germany) and a computer installed with software FIABOLO were used. The Clark-type oxygen electrode (model SME 1/4) from Elbau (Berlin, Germany) was composed of a Pt working electrode (diameter 0.5 mm) and an Ag/AgCl reference/counter electrode. A stirred measuring chamber with 1 ml volume was used for experiments.

### 2.2. Chemicals

3-hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30, 155 U $mg^{-1}$) was from BioScan Inc. (Shanghai, China), 3-hydroxybutyrate (HB) and β-nicotinamide adenine dinucleotide hydrate ($NAD^+$) were from Sigma (St. Louis, USA).