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ENZYMATIC REACTIONS FOR THE CALORIMETRIC DETECTION OF PHENOLIC COMPOUNDS

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Abstract

This study furnished results on the enzymatic detection of phenolic compounds by means of a miniaturized heat-flow calorimeter (IC-calorimeter). Two enzymes were used: tyrosinase and peroxidase. Additionally to the investigations with the IC-calorimeter, measurements were carried out with a classical reaction calorimeter (LKB 8700) for the very slow reactions with tyrosinase. By way of contrast, the reactions with peroxidase are fast and seem more suitable for sensor application. The detection limit for the investigated phenolic compounds is of the order of 1 mmol l^{-1}.

Keywords: enzyme sensor, IC-calorimeter, peroxidase, phenolic compounds, tyrosinase

Introduction

In recent years, numerous efforts have been made to investigate chemical reactions that can be utilized for the detection of pollutants with chemical sensors. There have been numerous suggestions as concerns enzymatic reactions for the specific determination of selected substances in complex mixtures. Most of them are applied to chemical sensors with an electrochemical or optical detection principle.

Soil and water in the area surrounding plants are often contaminated with toxic phenolic substances. Different phenolic compounds play important roles in the chemical industry for the production of a great variety of organic materials. They are present, for instance, in the production of plastics, insecticides, pharmaceuticals, paper and dyes, in oil refining, and in coal conversion [1–3].

Different enzymes and enzymatic reactions are suitable for the specific detection of phenolic substances. A large number of investigations have been carried out with tyrosinase for the determination of phenol, catechol and their derivatives [1, 2, 4–10]. Tyrosinase exhibits the activities of monophenol-mono-oxygenase (EC 1.14.18.1) and catechol-oxidase (EC 1.10.3.1). The former catalyzes the oxidation of monophenols to ortho-diphenols (catechols) and the latter the oxidation of diphenols to quinones. Because of the instability of these quinones in aqueous solution, polymerization occurs. The run of the reaction and the often insoluble polymerization products are largely unknown.

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Tyrosinase generally displays low substrate specificity. It catalyzes the oxidation of (para-)substituted monophenols and certain diphenols, especially ortho-diphenols, too [1, 3, 11–13].

Because of the problems with the run of reactions catalyzed by tyrosinase, the use of another enzyme for the determination of phenols appears of interest. Peroxidase (POD) catalyzes the dehydrogenation of a large number of organic compounds, such as phenols, aromatic amines and hydroquinones. The essence of the reaction catalyzed by POD is the transfer of hydrogen from a donor to hydrogen peroxide. The reaction follows a complex multistage mechanism, which depends in detail on the investigated substrate and the type of POD used. The radical transition products often undergo complicated secondary reactions. For instance, guaiacol used as a hydrogen donor gives octadehydrotetraguaiacol. In some other cases, the final products remain unknown [12, 14]. It therefore seems valuable to obtain more information on the usefulness of POD for the determination of phenolic compounds.

The aim of the present work was to investigate the possibilities and the limits for the application of enzymatic reactions with tyrosinase or POD for the detection of selected phenolic compounds (unsubstituted phenol, catechol, *p*-cresol and guaiacol). A miniaturized calorimeter with an integrated circuit as the essential part (IC-calorimeter) was applied to study the run of the enzymatic reactions; it may be considered a sensor with calorimetric detection. The silicon chip contains a sensitive thermopile for the temperature measurement and an electrical resistance for the thermal calibration, as parts of the integrated circuit. The calorimetric system is completed by a microlitre syringe for the addition of a second component in batch mode to start the investigated reaction on the chip surface. From the measured heat flow signal of the IC-calorimeter with an extremely low time constant, information is derived concerning the thermodynamic and kinetic parameters of the reaction, the concentration of the substrate or the enzyme activity.

In addition to the investigations with the IC-calorimeter, measurements with a classical reaction calorimeter (LKB 8700) were carried out for comparison and supplementation of the reaction parameters. For chemical reactions with a very long reaction period, such a calorimeter with a large time constant is more suitable.

Experimental

IC-calorimeter

Figure 1 schematically outlines the main parts of the calorimeter. Details of the IC-calorimeter may be found in previous publications [15, 16]. The advantages of such a miniaturized calorimeter include the considerable time-saving of the measurements, the small sample mass necessary for investigations and the excellent dynamic behaviour.

The calorimeter consists of two axially connected cylindrical aluminium blocks. The integrated circuit is located in the centre of the blocks. The calorimeter operates at room temperature without temperature regulation. An axial hole in the upper cylin-

der serves for the mounting of a Hamilton syringe, which holds the second reactant before the addition process. The moist ring attached to the top of the interior of the calorimeter adjusts the vapour pressure and reduces the rate of evaporation from the sample. Typical parameters of the detection system are: sensitivity 2.5 V W^{-1}, power resolution 0.1 μW and time constant 0.15 s.

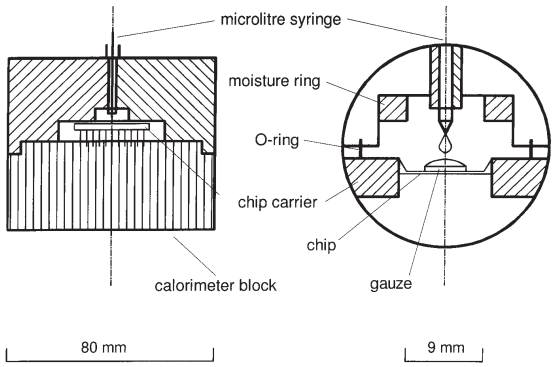

Fig. 1 Schematic illustration of the IC-calorimeter used

All measurements were carried out in the same way: A volume (3 μl) of substrate solution was placed on the chip before the device was closed. After the establishment of thermal equilibrium, the reaction was started by the addition of a drop (4.4 μl) of enzyme solution by means of the Hamilton microlitre syringe. The moisture ring was impregnated with the same substrate solution for preliminary saturation of the vapour chamber.

The evaluation of the heat-flow *vs*. time graphs followed with the aid of special computer programs.

LKB calorimeter

For comparison and supplementation of the results of IC-calorimetry, investigation were performed in a modified isoperibolic LKB 8700 calorimeter (producer LKB, Stockholm, Sweden). This calorimeter, with a time constant near 10000 s, is especially suitable for reactions with a long duration. The specification of the arrangement and the computer program for the data evaluation are described elsewhere [17]. All our measurements were carried out at a temperature of 298.15 K. The phenolic solution was placed in the calorimeter cell (80 ml) and the dissolved enzyme in the ampoule (1 ml).

Chemicals

Enzymes

Tyrosinase (EC 1.14.18.1) from mushrooms, 105 U mg^{-1} (Fluka); peroxidase (EC 1.11.1.7) from horse liver, 400 U mg^{-1} (Serva)

Substrates

Phenol, 1,2-dihydroxybenzene (catechol), 4-methylphenol (*p*-cresol), 2-methoxyphenol (guaiacol), hydrogen peroxide

Buffer

0.1 M phosphate buffer pH 6.9 according to Sörensen

Solutions

All solutions were prepared by using the same buffer, and the reagents used were of analytical grade (p.a.). The concentration of enzyme solutions was 2 g l^{-1}. The tyrosinase solution and the solutions of phenol or *p*-cresol can be stored at +5°C for some days, but the solutions of POD, catechol, guaiacol and hydrogen peroxide were prepared fresh every day.

For reactions with POD, the substrate solutions were prepared by mixing a phenolic stock solution with a hydrogen peroxide stock solution just before the investigation [14]. The concentration of hydrogen peroxide in these solutions was 19.8 mmol l^{-1} in all experiments.

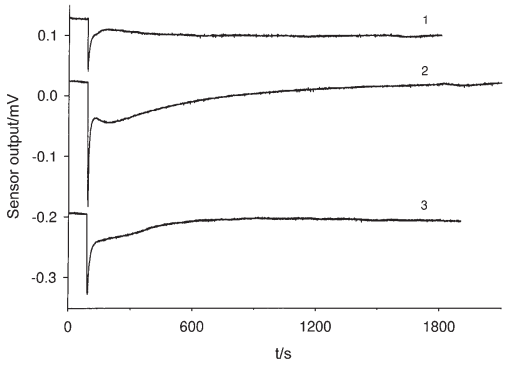

Fig. 2 Comparison of measurements with the IC-calorimeter for the tyrosinase-catalyzed decompositions of 1 – phenol, 2 – catechol and 3 – *p*-cresol (c_{phenol}≈11 mmol l^{-1})

Results and discussion

Results from measurements with the IC-calorimeter for the tyrosinase-catalyzed reactions of solutions of equal concentration (c=11 mmol l^{-1}) of different phenolic derivatives are given in Fig. 2. The shapes of the measured curves for the other concentrations are completely comparable. The narrow peak which appears directly after addition of the second component is caused by effects of addition, mixing and dilution. The run of the enzymatic reaction is very slow and there are great differences in the run of the reaction for the different phenolic derivatives. If unsubstituted phenol is

used as substrate, only a drift of the baseline over more than 30 min is observed. The assumption that this reaction is extremely slow was proved by measurements with the LKB 8700 calorimeter (see below).

The POD-catalyzed reactions of all investigated phenolic derivatives proceed much faster than the corresponding reactions with tyrosinase. This results in far higher and shorter peaks in the calorimetric curves, as can be seen, for example, in Fig. 3. The thermal effect is largely complete after 3–4 min. The evaluation of the curves is therefore more reliable and the scatter of the results from the measurements is much lower. For these reaction systems, no significant variation in reactivity of the different phenolic compounds was observed, as is described for tyrosinase. Overall, the system with POD seems more suitable for application as a calorimetric sensor.

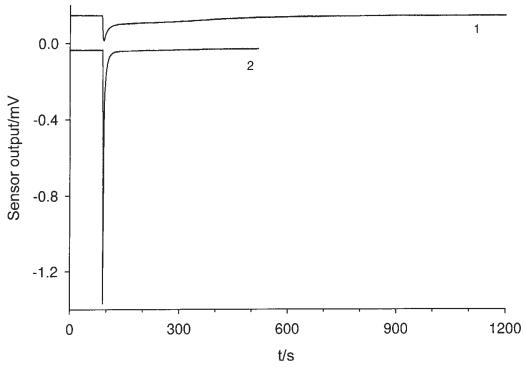

Fig. 3 Comparison of measurements with the IC-calorimeter for the decompositions of *p*-cresol catalyzed by 1 – tyrosinase and 2 – peroxidase ($c_{\text{p-cresol}}$≈11 mmol l^{-1})

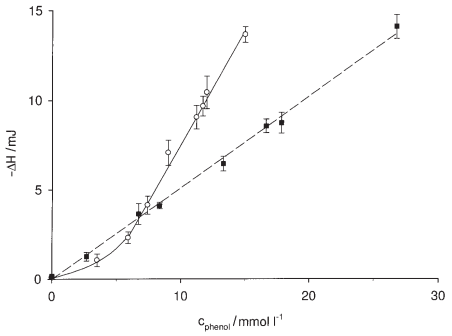

Fig. 4 Dependence of the reaction enthalpies of the tyrosinase-catalyzed reactions, determined with the IC-calorimeter, on the concentration of o – catechol or ■ – *p*-cresol

Additionally, we investigated the concentration dependence of the enthalpy ΔH with the IC-calorimeter. The results are shown in Figs 4 and 5. For all the studied systems, a linear increase in ΔH with increasing concentration of the phenolic derivative

was observed in a wide concentration range. Only the reaction of catechol with tyrosinase appears to be an exception. At concentrations lower than 6 mmol l^{-1}, the increase is not constant. For this unsubstituted diphenol, the measured extensive value of enthalpy is probably strongly influenced by the additional enthalpy of polymerization of the reaction product, and the degree of polymerization could be dependent on the concentration of the quinone formed.

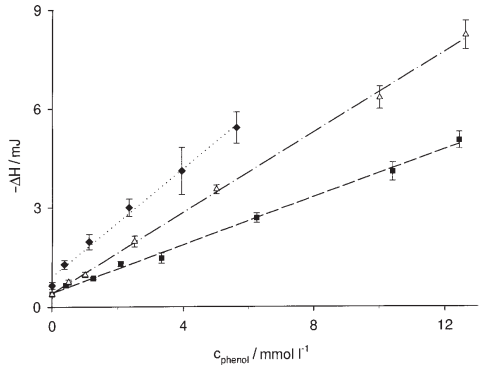

Fig. 5 Dependence of the reaction enthalpies of the peroxidase-catalyzed reactions, determined with the IC-calorimeter, on the concentration of ♦ – phenol, ∆ – guaiacol or ■ – *p*-cresol

The detection limit is near 1 mmol l^{-1} for all of the used phenolic compounds. Table 1 lists the numerical results for the slopes of the $\Delta H(c_{phenol})$ curves. They can be interpreted as analytical sensitivities, but not as reaction enthalpies, because of the undetermined reaction course.

Table 1 Results of measurements with the IC-calorimeter

Enzyme	Phenolic substrate	Sensitivity/kJ mol^{-1}
Tyrosinase	phenol	–
	catechol	–421±21
	p-cresol	–171±5
Peroxidase	phenol	–272±22
	guaiacol	–203±5
	p-cresol	–120±4

As we had problems in the investigation of the tyrosinase-catalyzed decomposition of phenol by means of the IC-calorimeter, we carried out experiments with an LKB 8700 calorimeter. In comparison with the IC-calorimeter, this device has a high time constant and better possibilities for the handling of the reaction system (mixing, stirring and gas-flow through the solution). Therefore, it is possible to observe a reaction over several hours, to change the reaction conditions, and to obtain first information on the kinetics.

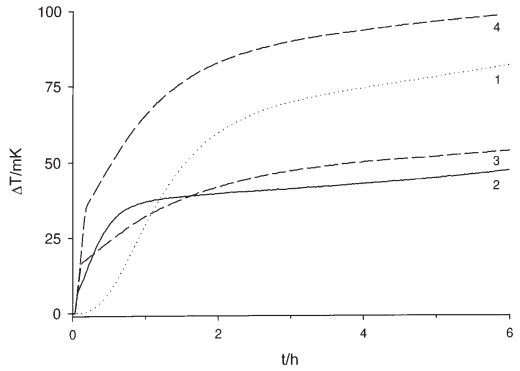

Fig. 6 Comparison of measurements with the LKB-calorimeter on the tyrosinase-catalyzed decompositions of 1 – 5.04 mg phenol, 2 – 3.50 mg catechol, 3 – 3.00 mg *p*-cresol and 4 – 5.00 mg *p*-cresol. The curves are corrected for the heat-flow

Results of the investigations with tyrosinase are given in the $\Delta T(t)$ curves in Fig. 6. The curves are corrected for the heat-flow, using a cooling constant, which was determined in calibration experiments under analogous conditions. The results confirm that the reactions with the different phenolic compounds are slow. The exothermic process continues during more than 6 h, as indicated by a continuing rise in temperature difference.

For unsubstituted phenol, the thermal effect is first appreciable approximately 5–10 min after addition of the tyrosinase solution. The reason for this could be that the commercially available enzyme used contains mainly native tyrosinase. This modification of the enzyme must be transformed into desoxytyrosinase by reaction with catechol or another 2-electron donor. Desoxytyrosinase is able to accept oxygen with the formation of oxytyrosinase, which can bind phenol for hydroxylation. Therefore, only a small proportion of the commercial tyrosinase is active from the very beginning and forms the needed catechol from phenol [18]. Thus, in a wider sense it is an autocatalytic process.

We tried to influence the reaction rate via the mass of enzyme, by using another charge of enzyme, and via the flow of gaseous oxygen through the solution, but this did not lead to marked changes. Chemical activation of the tyrosinase by the addition of small amounts of catechol or $FeCl_2$ as electron donor to the tyrosinase solution was likewise without success.

From the measurements with the LKB calorimeter on the decompositions of catechol and *p*-cresol with tyrosinase, it was possible to separate the effect of the enzymatic reaction (rapid) from that of the following reactions (slow, several hours). A break in the strongly rising part of the $\Delta T(t)$ curves indicates the end of the enzyme-catalyzed reaction. The ΔT value at this point depends on the mass of substrate, as is shown in Fig. 6, for example, for two different concentrations of *p*-cresol. The value of the molar reaction enthalpy evaluated on the basis of this finding from the measured curves agrees with the value expected for the formation of quinone from

phenol or catechol. Further, it appears that the contribution of the following reactions to the total enthalpy change in relation to that of the enzymatic reaction is higher for catechol than for *p*-cresol. This could be caused by different polymerisation behaviour in consequence of the different substituents.

Conclusions

By means of a miniaturized heat-flow calorimeter, the suitability of enzymatic reactions for the detection of different phenolic derivatives was investigated. The results obtained reveal that the use of tyrosinase for the quantitative thermal detection of several phenolic compounds involves significant problems. The run of the reactions is extremely slow and the evaluation of the measured curves is therefore problematic. The reactions with peroxidase seem more suitable for sensor application, because the thermal effects are short and consequently the scatter in the data is low. The limit of detection of the thermal system is of the order of 1 mmol l^{-1} for all the investigated phenolic compounds.

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