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Co-immobilization of oxalate oxidase and catalase in films for scavenging of oxygen or oxalic acid

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ABSTRACT

Oxalate oxidase has potential to act as an oxygen scavenger in active packaging to increase the shelf-life of food and beverages, while simultaneously producing the protective packaging gas carbon dioxide. This study shows that oxalate oxidase from barley can be immobilized with retained catalytic activity through entrapment in a latex polymer matrix. Conditions for formation of film containing oxalate oxidase have been evaluated as well as effects of storage and latex on enzyme activity, migration of enzyme in films, and the ability of the latex films to resist higher temperatures. Drying of enzyme-containing latex films at 75 °C prior to conditioning at 30 °C resulted in higher activity than drying solely at 30 °C, or drying at 95 °C or 105 °C followed by conditioning at 30 °C. Storage of films in air at 4 °C for 14 days did not negatively affect the enzymatic activity. Inclusion of catalase in films with oxalate oxidase effectively prevented release of hydrogen peroxide. The results suggest that the immobilized enzyme can successfully be used both as an oxygen scavenger and as an oxalic-acid scavenger.

1. Introduction

Oxalate oxidase (EC 1.2.3.4) catalyzes the conversion of oxalic acid and molecular oxygen to carbon dioxide and hydrogen peroxide (I).

$$HOOC{-}COO^- + H^+ + O_2 \rightarrow 2CO_2 + H_2O_2 \quad (1)$$

Oxalate oxidase has been found in fungi, bacteria, and plants like barley and wheat. Oxalate oxidase belongs to a large family of germin-like proteins that have been termed cupins because of their conserved β-barrel fold [1]. The active site, which contains a manganese ion, is found within the center of the β-barrel. The pH optimum of oxalate oxidase has been reported to be 3.8 [2] and the K_M value has been determined to 0.28 mM [3]. The sensitivity of oxalate oxidase towards inhibitory compounds has been studied previously [2–5].

Oxalate oxidase is mainly used in the analysis of the levels of oxalic acid in clinical samples, such as blood plasma and urine [1]. Analysis of oxalic acid in clinical samples is of relevance for patients with kidney stones, which mainly consist of crystallized calcium oxalate. Patients suffering from reoccurring kidney stones are often recommended a diet with low content of oxalic acid. Another approach is to treat patients suffering from primary and secondary hyperoxaluria with the oxalate-degrading bacterium *Oxalobacter formigenes* [6]. There are also problems with calcium oxalate precipitations in industry, for example in the pulp and paper industry [7]. Thus, one potential application for oxalate-degrading enzymes and microorganisms is to prevent formation and precipitation of calcium oxalate.

Another potential application for oxalate oxidase is as an oxygen scavenger. The most widely used type of active packaging today is based on the scavenging of oxygen. Oxygen-scavenging enzymes suggested for active packaging application today include glucose oxidase, laccase and ethanol oxidase (Table 1). The fact that oxalate oxidase could potentially scavenge both oxalic acid (to make the food healthier) and oxygen (to increase the shelf-life of food) makes it a very interesting enzyme in the field of active packaging.

For the production of enzyme-based active packaging, it is desirable to immobilize the enzyme onto any of the components of the packaging material. The simplest immobilization technique for active packaging would be entrapment of the enzyme in a dispersion coating. Glucose oxidase was recently successfully entrapped in a polymer-based dispersion coating for the use as an oxygen scavenger in active packaging [8]. In this work, we have investigated immobilization of oxalate oxidase in films through entrapment in a latex polymer matrix. The possibility to utilize

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Table 1
Oxygen scavenging enzymes suggested for packaging applications.

Oxygen-scavenging enzyme	Reaction catalyzed	Reference
Glucose oxidase	glucose + O_2 → gluconic acid + H_2O_2	Nestorson et al. [8]
Laccase	4Ar–OH + O_2 → 4Ar–O· + 2H_2O	Johansson et al. [28]
Ethanol oxidase	ethanol + O_2 → acetaldehyde + H_2O_2	Labuza and Breene [29]

oxalate-oxidase-based systems either as oxygen scavengers or as oxalic acid scavengers was explored. Furthermore, we also investigated the possibility to co-immobilize catalase to degrade the hydrogen peroxide produced in the reaction catalyzed by oxalate oxidase. The reaction catalyzed by catalase generates oxygen (II), but when both reactions (I and II) are combined they result in a net consumption of oxygen (III):

$$H_2O_2 \rightarrow H_2O + (1/2)O_2 \quad (2)$$

$$HOOC{-}COO^- + H^+ + (1/2)O_2 \rightarrow 2CO_2 + H_2O \quad (3)$$

To our knowledge this is the first study of oxygen-scavenging films based on oxalate oxidase and co-immobilization of catalase to prevent release of hydrogen peroxide.

2. Materials and methods

2.1. Preparation of free films

Oxalate oxidase from barley seedlings (0.71 U/mg; one unit (U) forms one μmol of hydrogen peroxide per min at pH 3.8 and 37 °C) (Sigma–Aldrich, Steinheim, Germany) was mixed with HPU70 latex (styrene butadiene co-polymer; Tg 6 °C; dry solids content 50.6%) (Styron Europe GmbH, Horgen, Switzerland), hereafter denoted as SB-latex, in a ratio of 416 g dry latex/g enzyme preparation. The mixture was incubated for 20 min at 4 °C with stirring to ensure homogeneous dispersion. Oxalic acid was added to a portion of the enzyme/latex dispersion to evaluate the need for having oxalic acid, one of the substrates of the enzyme, present directly in the film. For this experiment, 2.033 g of the enzyme/latex dispersion was mixed with oxalic acid to a final concentration of 20 mM. The pH of the dispersion without oxalic acid was 5.5, while the pH was 4.9 in the dispersion containing oxalic acid. The mixture was left to stir for an additional 15 min at 4 °C. The two dispersions with and without oxalic acid were coated onto the backside of silicone-treated release papers using a wire-wound bar (K202 Control Coater, RK Print Coat Instruments Ltd., Royston, UK) resulting in a nominal wet deposit of 60 μm. Unless otherwise indicated, the drying procedure [9] consisted of an incubation for 30 s at 105 °C in a ventilated oven followed by an incubation for 24 h at 30 °C and 50% relative humidity (RH) to ensure complete film formation. When substrate was present the extended drying was performed under nitrogen atmosphere to prevent pre-oxidation of the substrate. In order to evaluate the influence of the drying conditions on the enzyme activity, free films without oxalic acid were prepared also by (i) drying for 24 h at 30 °C and 50% RH, (ii) drying for 30 s at 75 °C followed by incubation for 24 h at 30 °C and 50% RH, and (iii) drying for 30 s at 95 °C followed by incubation for 24 h at 30 °C and 50% RH.

As a negative control, SB-latex without enzyme was coated onto the backside of a release paper as described above. The drying procedure for the negative control was 30 s at 105 °C followed by 24 h at 30 °C and 50% RH.

The films were stored at 4 °C until further analyzed. Prior to analysis, the films were peeled off from the release paper.

2.2. Enzyme activity measurements using oxygen electrode

The specific activity of the enzyme-containing films was evaluated using an oxygen electrode (Oxygraph system, Hansatech Instruments, Kings Lynn, UK). A small piece of film was weighed and added to the reaction chamber that contained 3 ml pre-heated (25 °C) reaction mixture. The reaction mixture for the films without substrate contained 2 mM oxalic acid pH 3.8 (the pH was adjusted to 3.8 with a solution of sodium hydroxide) and 20 mM succinic acid buffer (pH 3.8). The reaction mixture for the films with oxalic acid contained only 20 mM succinic acid buffer (pH 3.8). The weight of the films with oxalic acid corresponded to a maximum theoretical concentration of oxalic acid in the reaction chamber of 2 mM. The loss of activity due to immobilization was determined by comparing the specific activity of the enzyme before and after immobilization.

All measurements were performed in triplicates and the degradation of oxalic acid was measured during 10 min. The specific activity was calculated as μmol O_2 min^{-1} mg enzyme preparation^{-1}.

2.3. Film stability

The film prepared as negative control was used to investigate the stability of the latex films at higher temperatures when incubated in aqueous solutions. Three pieces of film were peeled off from the release paper and weighed. Two pieces of films were incubated, each separately, in 3 ml of deionized water; one at 65 °C and the other one at room temperature. The third piece was used as a control and was not incubated in water. The films were subsequently allowed to dry during 24 h at 30 °C and 50% RH. The weight of the films was determined before and after the treatment.

2.4. Migration of the enzyme

A film without substrate was used to evaluate if the enzyme migrates out from a film incubated in an aqueous solution for a short period of time. A small piece of free film was weighed prior to incubation for 10 min in 3 ml 20 mM succinic acid buffer (pH 3.8). The film was thereafter removed and the 3 ml succinic acid buffer solution was transferred to the oxygen electrode chamber. A volume of 12 μl 0.5 M oxalic acid pH 3.8 (the pH was adjusted to 3.8 with a solution of sodium hydroxide) was added to the oxygen electrode chamber resulting in an initial oxalic acid concentration of 2 mM. The concentration of oxygen was then monitored during 10 min. As a positive control, the activity of the same amount of film without substrate placed in the reaction chamber was measured according to the section "Enzyme activity measurements using oxygen electrode".

2.5. Storage of enzyme-containing films

The storage stability of enzyme-containing films without substrate was studied by measuring the activity (as described in Section 2.2) after 1, 6 and 14 days of storage at 4 °C in air atmosphere.

2.6. The effect of latex on enzyme activity

The potential inhibitory effect of SB-latex on the activity of oxalate oxidase was measured using the oxygen electrode. In all experiments, the reaction mixture contained 20 mM succinic acid,