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Immobilization of an enzymatic extract from *Penicillium camemberti* containing lipoxygenase and hydroperoxide lyase activities

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Abstract

An enzymatic extract from *Penicillium camemberti*, containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities, was immobilized on oxirane acrylic beads, Eupergit C and Eupergit C250L-iminodiacetic acid (IDA). The optimum pH for LOX activity was determined to be 4.0 and 6.0 for the free enzyme extract and 6.0 for the immobilized one, whereas that for the HPL activity was 4.0 and 6.0 for the immobilized and free extracts. The optimal reaction temperature for LOX activity was 30 and 55 °C for the free and immobilized enzyme extracts, respectively, whereas the HPL activity showed its optima at 45 and 30 °C, for the free and immobilized extracts, respectively. The immobilization of the enzymatic extract dramatically enhanced the thermostability of LOX and HPL activities. In term of enzymatic stability, the lyophilized immobilized extract showed that its HPL activity at 4 °C was more stable than that of LOX. The results indicated a decrease and an increase in enzyme efficiency for LOX and HPL activity, respectively, upon immobilization.

Keywords: Lipoxygenase; Hydroperoxide lyase; *Penicillium camemberti*; Immobilization; Eupergit

1. Introduction

Lipoxygenase (LOX) (linoleate: oxygen oxidoreductase; EC 1.13.11.12) catalyzes the dioxygenation of various polyunsaturated fatty acids (PUFAs) containing a 1(*Z*),4(*Z*)-pentadiene moiety into various regio-isomers of hydroperoxides of PUFAs, which can be subsequently cleaved by hydroperoxide lyase (HPL) into aldehydes and alcohols [1]. The sequential action of LOX and HPL enzymes results in the bioconversion of PUFAs acids into a wide variety of flavor compounds. Although there has been an increasing interest in the LOX/HPL biocatalyzed production of aroma compounds [2,3], the limited stability of these enzymes has restricted their biotechnological applications [4].

Immobilization offers a substantial enzymatic stabilization as well as the possibility of the reuseability of the biocatalyst in continuous packed-bed reactors [4]. Further benefits of immobilization, include the easy separation of the enzyme from its end products, which minimizes downstream processing costs [5]. The immobilization of an enzyme to a support is contingent on chemical bond formation between the functional groups of the immobilization support and those of the enzyme. Several types of immobilizations exist, including adsorptive, entrapment and crosslinking [5]. Covalent supports form bonds at a variety of attachment points on the enzyme, including $–NH_3^+$, $–COO^-$ and –SH groups [6,7]. Oxirane acrylic resins possess many oxirane groups that bind to the primary amines of the enzyme protein molecule [8]. Eupergit® supports are among the most used types of covalent supports and have been employed in the stabilization of different types of enzymes, often by multipoint attachment between the enzyme and the support [8]. Several studies have been carried out for the immobilization of enzymatic extracts, containing LOX [9,10] and HPL activities [6,7].

The overall objective of this study was to investigate the effect of immobilization on a LOX/HPL enzymatic extract, from *Penicillium camemberti*, using different supports. The specific objectives were to characterize the LOX/HPL activity in free and immobilized extracts, in terms of optimum pH,

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reaction temperature, thermostability, long-term stability and other kinetic parameters.

2. Materials and methods

2.1. Materials

Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was purchased from Nu-Chek Prep (Elysian, MN). Silica gel support was purchased from Silicycle (Quebec City, Qc), whereas Eupergit® C and Eupergit® C250L (oxirane acrylic beads) were offered as gifts from Rohm Pharma (Darmstadt, Germany). Dowex® 50WX4-200 (anionic ion-exchange resin), ethylenediamine (EDA) and iminodiacetic acid (IDA) as well as trishydroxy methylaminomethane (TRIS) were obtained from Aldrich (Milwaukee, WI). Xylenol orange [(3,3′-*bis*(*N*,*N*-di(carboxymethyl)aminomethyl)-*o*-cresol)] was purchased from Sigma Chemical Co. (St. Louis, MO). Mono- and dibasic potassium phosphate were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Culture growth and preparation of the enzymatic extract

P. camemberti was induced to sporulate, and the resultant spore suspension was counted, using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA) for the inoculation of liquid medium, according to the procedure outlined by Perraud and Kermasha [11]. After harvesting the biomass after 10 days of fermentation, it was filtered through cheesecloth and the mycelia were washed (2× 50 mL) with cold water (4 °C) followed by potassium phosphate buffer solution (pH 6.5, 0.1 M). The recovered mycelia were blended (5 mL of the phosphate buffer per 1 g biomass) and homogenized, using 0.45–0.50 mm diameter glass beads, in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2× 2 min. The LOX/HPL enzymatic extract was recovered by centrifugation (12,000 × *g*, 15 min) and concentrated by ultrafiltration (Amicon, 30 kDa NMWCO, 40 psi). All subsequent steps were performed at 4 °C, unless otherwise stated.

2.3. Preparation of oxirane acrylic supports

The investigated supports, including the covalent oxirane acrylic supports Eupergit® C and Eupergit® C250L, which were used as unmodified and modified supports with EDA and IDA, were prepared according to the procedure outlined by Mateo et al. [12]. The modification involved the suspension of the support (1 g wet weight) in 10 mL of EDA (5%, w/v) or 5 mL of IDA (1.8 M); the suspension was subjected to gentle stirring at 25 °C for 15 min and 5 h, respectively. The modified EDA and IDA supports were washed with deionized water.

2.4. Immobilization of LOX and HPL

The immobilization of LOX and HPL, expressed in the enzymatic extract from *P. camemberti*, was conducted at 4 °C, using 40 mg protein/g wet support. Potassium phosphate buffer solution (pH 6.5, 0.1 M) was used for all steps of immobilization, unless otherwise indicated. The immobilization on unmodified and modified supports was carried out, in conical 5 mL screw-cap tubes under mild agitation, using the phosphate buffer solution at 1.0 and 0.1 M concentration, respectively. After 18 h, the agitation was halted and the supernatants were recovered for protein determination. The supports, containing the immobilized enzymatic extract, were washed with 1× 15 mL of deionized water and 2× 15 mL of the phosphate buffer, where each wash solution was recovered for protein determination. The washed supports, containing the immobilized enzymatic extract, were re-suspended in the phosphate buffer solution (0.1 g wet support/mL) and assayed for LOX and HPL activities. Protein immobilization yield (%) was defined as the ratio of protein, immobilized onto a support (mg), divided by the initial protein content (mg) multiplied by 100. The retention of enzyme activity (%) was defined as the specific activity of LOX or HPL of the immobilized enzyme extract, divided by the specific activity of LOX or HPL of the free extract and multiplied by 100.

2.5. Substrate preparation

For LOX studies, linoleic acid was used as substrate; linoleic acid stock solution was prepared at a concentration of 4.0 mM in the appropriate buffer solutions (0.1 M), according to the procedure outlined by Perraud et al. [13]. For HPL studies, 10-hydroperoxide of octadecadienoic acid (10-HPOD) was used as substrate; 10-HOPD was obtained by the photo-oxidation of linoleic acid and purified by solid phase extraction followed by a prepative normal phase high-performance liquid chromatography (NP-HPLC), according to the procedure outlined by Kermasha et al. [14].

2.6. LOX assay of free and enzymatic extracts

For the LOX assay of the free enzymatic extract, it was initiated by the addition of 180 μL of the enzyme suspension (1.5 mg protein/mL) to 0.6 mL of substrate solution (4.0 mM), and the total volume was adjusted 1.5 mL with sufficient quantity of the buffer solution. The LOX assay for the immobilized enzymatic extract was initiated by the addition of 0.6 mL of immobilized enzyme suspension (0.1 g support/mL) to 1 mL of substrate (4.0 mM), and the total volume was adjusted 2.6 mL with sufficient quantity of the buffer solution.

The LOX assays for the free and immobilized enzyme extracts were carried out at 25 °C, under mild stirring, for 12 and 35 min, respectively. Aliquots of the reaction homogenate (0.1 mL) were taken at selected time intervals and were immediately added to 1 mL of xylenol orange reagent solution, which was prepared as a mixture of deionized/degassed water, ferrous sulfate (0.25 mM), perchloric acid (85.0 mM) and xylenol orange salt (0.1 mM) [15]. The absorbance of the reaction mixture was measured after 20 min of color development at 560 nm (10-HPOD; MEC 18,765 M^{-1} cm^{-1}), using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc.; San Raman, CA). LOX specific activity was defined as nmol of conjugated