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Catalytic properties of maltogenic α-amylase from *Bacillus stearothermophilus* immobilized onto poly(urethane urea) microparticles

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ABSTRACT

The immobilization of maltogenic α-amylase from *Bacillus stearothermophilus* (BsMa) onto novel porous poly(urethane urea) (PUU) microparticles synthesized from poly(vinyl alcohol) and isophorone diisocyanate was performed by covalent attachment to free isocyanate groups from PUU microparticles, or by physical adsorption of enzyme onto the surface of the carrier. The influence of structure, surface area and porosity of microparticles on the catalytic properties of immobilized BsMa was evaluated. The highest efficiency of immobilization of BsMa was found to be 72%. Optimal activity of immobilized BsMa was found to have increased by 10 °C compared with the native enzyme. Influence of concentration of sodium chloride on activity of immobilized BsMa was evaluated. High storage and thermal stability and reusability for starch hydrolysis of immobilized enzyme were obtained. Immobilized BsMa has a great potential for biotechnology.

1. Introduction

Amylases are widely present in microorganisms, plants and animals, and have found applications in numerous industries, mainly starch liquefaction for saccharification (Tavano, Fernandez-Lafuente, Goulart, & Monti, 2013). Although starch liquefaction can be accomplished via chemical processes, enzymatic hydrolysis can be performed under mild conditions, and could avoid the extreme conditions required by chemical treatments. Furthermore, the enzymatic process does not produce water pollution, which is very common in chemical processes (Tavano et al., 2013; Van der Maarel, van der Veen, Uidehaag, Leemhuis, & Dijkhuizen, 2002). Maltogenic α-amylase is a very important enzyme in the dairy industry that catalyses the conversion of starch into maltose which is an important sugar for many applications in the food and pharmaceutical industry (Derde, Gomand, Courtin, & Delcour, 2013). Maltogenic α-amylase from *Bacillus stearothermophilus* (BsMa) (EC 3.2.1.133) is an exo-acting enzyme, also known as glucan 1,4-α-maltohydrolase, which randomly cleaves off 1,4-α-D-glucosidic linkages in starch and other big carbohydrate molecules to produce shorter oligomaltose molecules, and which are hydrolysed from the non-reducing end to a final product of α configuration maltose (Leman, Goesaert, Vandeputte, Lagrain, & Delcour, 2005; Sharron & Elliott, 1969). Also, BsMa is used in transglycosylation reactions (Cha et al., 1998; Derde et al., 2013; Gaouar, Aymard, Zakhia, & Rios, 1997; Viskantiene, Budriene, Ramanaviciene, & Dienys, 2010).

Immobilization of enzymes is a very effective alternative in overcoming the problems of instability, expensively, sensitivity and repetitive use (Krajewska, 2004). Enzyme immobilization methods basically include adsorption to insoluble materials, entrapment in a polymeric matrix, encapsulation, crosslinking with a bifunctional reagent, or covalent linking to insoluble carriers (Miletic, Nastasovic, & Loos, 2012; Sheldon, 2007). The use of an immobilized enzyme permits the control of the reaction, by simple filtering or centrifugation of the enzyme to stop the reaction. Also, immobilized enzymes can be used several times or reused over long periods of time in reactors and, in addition to that, some critical enzyme properties have to be improved like stability, activity, inhibition by reactions products (Miletic et al., 2012; Sheldon, 2007).

Initially, BsMa was immobilized onto the silica carrier which was activated with glutaraldehyde (Kang, Kim, Kim, & Park, 1997). Further BsMa was immobilized onto porous and nonporous particles, in which one carrier was selected from the group consisting of phenolic, acrylic and polystyrene resin, (Duflot & Fouache, 2001). After this patent, the investigation of BsMa immobilization onto chitosan microparticles by covalent binding was carried out by our group some time ago (Zubriene et al., 2003). Good thermal stability of BsMa immobilized onto glutaraldehyde activated

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chitosan microparticles was obtained. Our research group has also explored the immobilization of BsMa onto polyurethane (PU) (Budriene, Romaskevic, Pielichowski, & Pielichowski, 2007; Romaskevic et al., 2010) and PUU poly(urethane-urea) (Straksys, Kochane, & Budriene, 2013) derivatives by covalent fixation. High efficiency of immobilization of BsMa was obtained when PU microspheres were synthesized using an initial molar ratio of 4,4′-diphenylmethane diisocyanate and 1,4-butanediol = 2:1 in chlorobenzene medium (Budriene et al., 2007). Also, good efficiency of immobilization of BsMa was shown on PU microparticles which was obtained from poly(vinyl alcohol) (PVA) and 1,6-hexamethylene diisocyanate (Romaskevic et al., 2010) and on PUU microparticles from PVA and a blend of diisocyanates (1,6-hexamethylene diisocyanate and 2,4-toluene diisocyanate). However, the variety of carriers is limited (Straksys et al., 2013).

Our earlier investigation of synthesis and properties of porous poly(urethane urea) PUU microparticles from poly(vinyl alcohol) (PVA) and isophorone diisocyanate (IPDI) (Straksys, Kochane, & Budriene, 2015) motivated us to study their application as carriers for the immobilization of maltogenic α-amylase from *Bacillus stearothermophilus* (BsMa). The aim of this study was to evaluate enzymatic activity, stability and reusability of BsMa immobilized onto novel PUU microparticles. The influence of properties of the obtained PUU microparticles, such as surface area and porosity, on the efficiency of immobilization of BsMa was investigated. Effects of ionic strength and storage time on relative and residual activities of immobilized BsMa onto PUU microparticles were evaluated. In addition, the reusability of immobilized maltogenic α-amylase preparations was examined through repeated batch experiments with the purpose to use for starch hydrolysis.

2. Materials and methods

2.1. Materials

Isophorone diisocyanate (IPDI) was purchased from Sigma-Aldrich, Germany. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, France. Acetone was purchased from Reachem Slovakia, Slovakia. Diethyl ether was purchased from LACH-NER, Czech Republic. Sodium chloride was purchased from Aldrich, Germany. Potato starch was purchased from Beroxfood, Germany. Poly(vinyl alcohol) (PVA, M_w 100,000, degree of hydrolisation 86–89 mol%, viscosity (4% in water, 20 °C) is 34–45 mPa s) was purchased from Fluka, Switzerland. PVA was dried under vacuum at 60 °C for 24 h and kept in a desiccator. All other reagents were used as received.

Maltogenic α-amylase (EC 3.2.1.133) (4000 U/ml) from *Bacillus stearothermophilus* (BsMa) and α-amylase (EC 3.2.1.1) (25,000 U/ml) from *Bacillus subtilis* were obtained from Novozymes, Denmark. Commercial enzyme preparations were used as purchased without additional purification.

2.2. Preparation of liquefied starch solution

Liquefaction of starch was performed according to this procedure: 100 ml of 5% potato starch suspension in 0.1 M sodium citrate buffer (pH = 5.0) was stirred at 90 °C for 5 min. After starch pasting, the suspension was cooled to 40 °C and 0.5 ml (250 U) of α-amylase from *Bacillus subtilis* was added. The starch solution was stirred at 40 °C for 3 min and after that enzyme was inactivated by heating for 30 min in a boiling water bath (Romaskevic et al., 2010). Dextrose equivalent (DE) of liquefied starch (substrate) was about 6%. The dextrose equivalent is a percentage measure of the extent of starch hydrolysis, which is expressed as the reducing power of the substance, i.e. glucose has a DE of 100%, maltose 50%, whereas starch dextrose equivalent is 0%. DE was calculated from the amount of reducing sugars produced in the substrate determined by the Neocuproine method (see Section 2.5) (Latimer, 2012). The DE was determined as:

$$DE(\%) = \frac{[\textit{Reducing Sugars in substrate},\ \text{mg/ml}]}{[\textit{Substrate},\ \text{mg/ml}]} \cdot 100$$

2.3. Synthesis of PUU microparticles

PUU microparticles were prepared according to the previous reported method (Straksys et al., 2015). Briefly, PVA was dissolved in dimethyl sulfoxide/water (99/1% vol) solution. The initial concentration of PVA was 0.06 M (mole of repeating units of PVA). The solution was loaded into a three-necked flask which contained a magnetic stirrer and reflux condenser with an attached drying tube and an argon gas inlet. IPDI was added and reaction was carried out at 60–160 °C for 60–180 min. The initial molar ratio of PVA and IPDI was varied from 1:2.0 to 1:4.0. Obtained PUU microparticles were precipitated into an acetone/diethyl ether mixture (1:1) and immediately used for immobilization of BsMa.

2.4. Immobilization of the enzyme

Immobilization of BsMa was carried out in 0.1 M citrate buffer (pH = 5.0) according to the previous reported method (Romaskevic et al., 2010). Briefly, the mixture of 0.38 ml (380 U) of the BsMa solution, 5 ml of 0.1 M sodium citrate buffer (pH = 5.0) and 1.75 g of PUU microparticles (immediately after synthesis, see Section 2.3) was stirred at 40 °C for 30 min and then left at 4 °C overnight. Next day the immobilized enzyme was thoroughly washed several times with buffer and catalytic activity of immobilized BsMa was determined (see Section 2.5).

2.5. Determination of enzymatic activity of BsMa

The enzymatic activity of native and immobilized BsMa onto carrier (0.1 g) was determined by a reaction with 10 ml of 5% liquefied potato starch solution and incubating the mixture at 40 °C for 20 min. In the case of native BsMa, 1 ml of BsMa solution diluted 160 times was used. The reaction was stopped by filtering the PUU microparticles from the reaction mixture and by heating in a boiling water bath for 10 min (Romaskevic et al., 2010). Activity unit of native or immobilized BsMa was defined as the amount of enzyme which under standard conditions (at 40 °C, pH = 5.0) produced 1 μmol of reduced sugars per minute. The amount of reducing sugars produced in this reaction was determined spectrophotometrically by the Neocuproine method (Bittner & Manning, 1967). The protein content in the native enzyme solution or left in the solution after immobilization was assayed by the bicinchoninic acid method (BCA kit) (Smith et al., 1985). Four separate measurements of the native and immobilized BsMa were performed to check the reproducibility of the data.

Efficiency of immobilization (EI) was defined as the specific activity of immobilized BsMa in percentage from the activity of native enzyme used for immobilization.

$$EI(\%) = \frac{\textit{activity of immobilized enzyme}}{\textit{activity of native enzyme}} \cdot 100$$

Yield of immobilization by protein (YP) was defined as protein quantity of immobilized enzyme in percentage from the quantity of protein of native enzyme used for immobilization.

$$YP(\%) = \frac{\textit{Protein quantity of immobilized enzyme}}{\textit{Protein quantity of native enzyme}} \cdot 100$$