Bioresource Technology 115 (2012) 158–163

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

A novel and efficient method for the immobilization of thermolysin using sodium chloride salting-in and consecutive microwave irradiation

Feifei Chen [a,b], Fangkai Zhang [a,c], Fangchuan Du [a,c], Anming Wang [a,*], Weifang Gao [a], Qiuyan Wang [a], Xiaopu Yin [a], Tian Xie [a]

[a] Research Center for Biomedicine and Health, Hangzhou Normal University, Hangzhou 310012, PR China
[b] College of Material, Chemistry and Chemical Engineering, Hangzhou Normal University, No. 222, Wenyi Road, Hangzhou 310012, PR China
[c] College of Biological and Environmental Sciences, Hangzhou Normal University, Hangzhou 310012, PR China

ARTICLE INFO

Article history:
Available online 25 November 2011

Keywords:
Salting-in
Microwave irradiation
Immobilization
Thermolysin
Organic solvent

ABSTRACT

Sodium chloride salting-in and microwave irradiation were combined to drive thermolysin molecules into mesoporous support to obtain efficiently immobilized enzyme. When the concentration of sodium chloride was 3 M and microwave power was 40 W, 93.2% of the enzyme was coupled to the support by 3 min, and the maximum specific activity of the immobilized enzyme was 17,925.1 U mg^{-1}. This was a 4.5-fold increase in activity versus enzyme immobilized using conventional techniques, and a 1.6-fold increase versus free enzyme. Additionally, the thermal stability of the immobilized thermolysin was significantly improved. When incubated at 70 °C, there was no reduction in activity by 3.5 h, whereas free thermolysin lost most of its activity by 3 h. Immobilization also protected the thermolysin against organic solvent denaturation. The microwave-assisted immobilization technique, combined with sodium chloride salting-in, could be applied to other sparsely soluble enzymes immobilization because of its simplicity and high efficiency.

1. Introduction

Thermolysin (EC 3.4.24.27) is a thermostable, neutral metalloproteinase widely used in industry for the production of bioactive peptides through bond formation or protein hydrolysis (Kusano et al., 2010; Di Bernardini et al., 2011). Its most extensive use is in peptide preparation of N-carbobenzoxy L-Asp-L-Phe methyl ester (ZDFM) by coupling N-carbobenzoxy L-Asp (ZD) and L-Phe methyl ester (PM), a precursor of the artificial sweetener aspartame. Aspartame is 200 times sweeter than sucrose, a product of N-carbobenzoxy L-Asp and L-Phe methyl ester (Nagayasu et al., 1994a,b). The expression, purification, and production of recombinant thermolysin have been challenges, and reducing enzyme cost is an important subject in the production of ZDFM (Inouye et al., 2007).

The availability of an immobilized enzyme catalyst that has improved activity and stability is expected to reduce cost (Cao, 2006). In addition, immobilization can allow for the use of enzymes in different solvents, at extreme pH and temperatures and exceptionally high substrate concentrations (Nakanishi et al., 1985; Wang et al., 2011). There have been reports on the immobilization of thermolysin using adsorption (Xin and Si, 2010), covalent linkage (Kitano et al., 1996) and other methods (Persichetti et al., 1995). However, because of its low solubility (1.0–1.2 mg mL^{-1}) (Tatsumi et al., 2007) which resulted in aggregates and small number of amino side groups (⩽9) (Nakanishi et al., 1985), enzyme loading (Xin and Si, 2010) and relative activity (Kitano et al., 1996; Hoshino et al., 1997) of the immobilized enzyme is not efficient; therefore, highly efficient thermolysin immobilization has been a challenge both in the laboratory and on an industrial scale.

In the present study, we dissolve and disperse thermolysin in an immobilization mixture by salting-in effect and efficiently immobilize it in mesocellular siliceous foam (MCFs) support materials under consecutive irradiation at low temperature. Sodium chloride was used to disperse the aggregates and increase the enzyme solubility (Inouye et al., 1998) in the immobilization mixture, and consecutive microwave irradiation at low temperatures was used to speed the transport and accelerate the immobilization process. The effects of microwave irradiation power and time on the coupled yield and relative activity for the immobilized enzyme were determined. Thermal stability and resistance to organic solvents were also examined.

2. Methods

2.1. Materials

Crystalline thermolysin (1 X crystallized) from *Bacillus thermoproteolyticus rokko* (9050–18,100 U mg^{-1}) was purchased from

* Corresponding author. Address: Research Center for Biomedicine and Health, Hangzhou Normal University, No. 222, Wenyi Road, Hangzhou 310012, PR China. Tel.: +86 571 28861623; fax: +86 571 28865630.
E-mail address: anmwang@hotmail.com (A. Wang).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.11.059

Sigma–Aldrich. Triblock copolymer poly(ethylene glycol)-block-poly, (propyleneglycol)-block-poly(ethylene glycol) (P123) and tyrosine were also purchased from Sigma–Aldrich. N-Carbobenzoxy L-Asp-L-Phe methyl ester (ZDFM) was purchased from Hanhong Chemical Technology Co. Ltd. 1,3,5-Trimethylbenzene (TMB), ammonium fluoride, p-benzoquirone, sodium chloride, casein, ethyl acetate, and *tert*-amyl alcohol, all of which were of the highest grade commercially available, were provided by Sinopharm Chemical Reagent Co. Ltd. Other chemicals were of analytical grade and provided by Sinopharm Chemical Reagent (Shanghai, Sinopharm). Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

2.2. Microwave-assisted immobilization of thermolysin

MCFs support were prepared, characterized and functionalized as previously described (Schmidt-Winkel et al., 1999; Wang et al., 2011). Ten milligrams of functionalized MCFs were incubated in 3 ml of cross-linking agents at room temperature for 2 h, washed twice with an alcohol solution (20%, v/v), followed by distilled water. For the MCFs functionalized with 3-glycidoxypropyltrimethoxysilane, the functionalized support was used without crosslinking agent activation. The microwave-assisted immobilization of enzymes was performed in a commercial microwave reactor system (Discover, CEM, USA). The temperature in the reaction system is controlled by an optical fiber sensor. Ten milligrams of activated MCFs were redispersed in 3 ml of a 0.02 M MES–NaOH buffered solution (pH 7.0) containing a suitable amount of thermolysin, NaCl, $CaCl_2$ and $ZnCl_2$. The container with this mixture was microwave-irradiated by consecutive, focused microwaves for 3 min at low temperature for the immobilization reaction to occur. The immobilized enzyme was separated and washed using the same buffer until no protein could be detected in the supernatant. The amount of enzyme remaining in the supernatant was measured by the Bradford assay.

For the non-microwave-assisted enzyme immobilization, conventional methods were used. Briefly, the activated support was redispersed in 3 ml of a 0.02 M MES–NaOH buffered solution (pH 7.0) containing a suitable amount of thermolysin. The mixture of MCFs and enzyme solution was incubated in an ice-bath for 20 h with constant stirring. After immobilization, the immobilized enzyme was separated and rinsed using a 0.02 M MES–NaOH buffer (pH 7.0), and the amount of enzyme remaining in the supernatant was quantified by the Bradford assay.

2.3. Enzyme activity assays

The specific activity of thermolysin in casein hydrolysis was determined using a slightly modified version of a previously described method (Tatsumi et al., 2007). The immobilized thermolysin was redispersed in 132 ml of a 40 mM Tris–HCl (pH 7.5) buffered solution, and 0.5 ml was added to 1.5 ml of a solution containing 1.33% (w/v) casein and 40 mM Tris–HCl (pH 7.5) and incubated at 37 °C for 10 min. The reaction was stopped by adding 2 ml of a solution of 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid and incubating at 37 °C for 20 min. The reaction mixture was filtered, and the optical absorption of the filtrate was measured at a wavelength of 275 nm. One unit of enzymatic activity was defined as the amount of enzyme producing 1 μg of L-tyrosine in 1 min from casein at 37 °C. The coupled yield, relative activity and activity yield after coupling were defined as follows:

$$\text{Coupled yield}\,(\%) = \frac{(A-B)}{A} \times 100$$

$$\text{Activity yield}\,(\%) = \frac{C}{A} \times 100$$

$$\text{Relative activity}\,(\%) = \frac{C}{(A-B)} \times 100$$

In the above equations, A represents the total activity of the enzyme added in the initial immobilization solution, B is the activity of the same amount of enzyme in supernatant after the immobilization procedure, and C is the activity of the immobilized thermolysin.

2.4. Determination of the K_m and V_{max} for thermolysin hydrolyzation of the ZDFM substrate

The kinetic constant, K_m, and the V_{max} values of the free or immobilized thermolysin were determined by measuring the initial reaction rates with ZDFM as the substrate (Kusano et al., 2006; Tatsumi et al., 2007) at 25 °C in a 40 mM Tris–HCL buffered solution (pH 7.5) containing 3 M NaCl and 10 mM $CaCl_2$. Six concentrations of ZDFM, ranging from 0.2 to 1.0 mM, were used to determine the reaction rates. The K_m and V_{max} were calculated by double reciprocal Lineweaver–Burk plots using the initial rate of the reaction data.

$$-\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

[S] represents the substrate concentration, and V and V_{max} represent the initial and maximal reaction rates, respectively. K_m is the Michaels constant.

2.5. Thermo-stability of thermolysin preparations in aqueous and organic solvents

To evaluate the thermo-stability of the thermolysin preparations, both free and immobilized enzyme were transferred into the 40 mM Tris–HCl buffered solution (pH 7.5) containing 0.1 M NaCl and 10 mM $CaCl_2$, and incubated at 70 °C and 80 °C for different time periods. Samples in the suspension were periodically withdrawn, and their remaining activities were assayed as described above. Thermostability was defined as the residual activity of the immobilized derivatives or the soluble enzyme.

The organic solvent course of irreversible inactivation was measured by incubation of the enzyme at 70 °C for 2 h in the presence of different solvent concentrations. The aqueous buffer was a 40 mM Tris–HCl buffered solution (pH 7.5) containing 0.1 M NaCl and 10 mM $CaCl_2$. Ethyl acetate and *tert*-amyl alcohol, which are vital solvents used in peptide synthesis, were selected as the organic solvents. The concentrations of organic solvent ranged from 5% to 80% (v/v). After the incubation, the organic solvent was removed, and the remaining activity was assessed.

3. Results and discussion

3.1. Effects of salts on the activity of immobilized thermolysin using the microwave-assisted method

To disperse thermolysin well in the immobilization mixture to favor the immobilization in mesoporous support, some salts such as NaCl, $ZnCl_2$ and $CaCl_2$ were added in to the mixture to examine their effectiveness on the activity of immobilized thermolysin. The results show a dramatic increase in the relative activity of the immobilized thermolysin during casein hydrolysis in an immobilization mixture containing 20 mM $ZnCl_2$ and 3 M NaCl in the microwave-assisted immobilization (Fig. 1). The maximum relative activity reached 82.9%, which was 7.3-fold and 3.1-fold greater than that of the immobilized enzyme derived from the immobilization mixture with 5 mM $ZnCl_2$ and 20 mM $ZnCl_2$, respectively. However, only 4.6% of the relative activity for the immobilized thermolysin was detected when the immobilization mixture contained no salts, including $ZnCl_2$, $CaCl_2$ or NaCl. Compared to NaCl and $ZnCl_2$, $CaCl_2$ provides no detectable effect on the immobilized