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

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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 metallo-proteinase 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 thermo-proteolyticus rokko (9050–18,100 U mg⁻¹) was purchased from...