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Evaluation of cross-linked enzyme aggregates of *Lactobacillus* cell-envelope proteinases, for protein degradation

*Dominic Agyei, Lizhong He**

Department of Chemical Engineering, Monash University, Wellington Road, Clayton, Victoria 3800, Australia

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ABSTRACT

Enzymatic hydrolysis is a widely used approach to improve the functional, nutritional and physiological properties of food proteins. In this study, cross-linked enzyme aggregates (CLEAs) have been prepared from cell-envelope proteinases (CEPs) of *Lactobacillus delbrueckii* subsp. *lactis* 313 and their proteolytic properties have been evaluated using several food proteins. We have optimized cross-linking conditions including ammonium sulphate concentration, incubation temperatures, agitation speed, glutaraldehyde cross-linker concentration, reaction time and the addition of proteic feeders. Particularly, the presence of BSA improves retained activity of cross-linked CEP aggregates (CLCEPAs) from 21.5% to 40.9%. Blocking unreacted cross-linking groups on aggregates is important to enhance recyclability of CLCEPAs. CLCEPAs had attractive thermal stability at 50 °C and it showed enhanced catalytic activity over long-term storage after lyophilization. We have demonstrated that CLCEPAs has proteolytic properties on different food proteins including complex (chicken egg albumin, skimmed-milk protein), fractionated (bovine casein, whey protein isolate), and purified (bovine serum albumin) proteins. Being the first report of CLEAs from lactobacilli CEPs, this study demonstrates the feasibility of using LDL 313 CLCEPAs for degradation of various food proteins.

1. Introduction

Food proteins and their hydrolysates play key functional and physiological roles in food processing operations or in living body systems. Enzymatic hydrolysis continues to be one of the most widely used approaches for improving the functional, nutritional and physiological properties of food proteins. For technological and industrial applications, proteolytic enzymes must possess certain key requirements such as good stability and activity over a broad range of desired reaction conditions (e.g. extreme pH, elevated temperature, organic solvents, mechanical stress) (Brady and Jordaan, 2009; Chen et al., 2006). These sets of requirement retard the application of soluble enzyme in many industrial syntheses and processes.

Enzyme immobilization has been shown to improve enzyme stability and recyclability, thereby overcoming the challenges encountered with the use of soluble enzymes. Traditional methods of enzyme immobilization include physical adsorption, covalently binding to a carrier support, encapsulation and cross-linking (Zhao et al., 2013). Among these methods there has been an increased interest in cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) which are two attractive carrier-free immobilized

Abbreviations: CEPs, cell-envelope proteinases; CLEAs, cross-linked enzyme aggregates; CLCEPAs, cross-linked CEP aggregates; CLECs, cross-linked enzyme crystals; LDL 313, *Lactobacillus delbrueckii* subsp. *lactis* 313; CLCEPAs-BSA, cross-linked CEP aggregates prepared with BSA; OPA, *o*-phthaldialdehyde; DH, degree of hydrolysis; SMP, skimmed milk proteins; WPI, whey protein isolate; CEA, chicken egg albumin.

* *Corresponding author*. Tel.: +61 3 990 53437.
E-mail address: lizhong.he@monash.edu (L. He).
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enzymes systems (Sheldon, 2011). Nevertheless, CLECs have the drawback of cost and tedious crystallization procedures, mainly because very pure enzyme preparations are required for the crystallization step (Wilson et al., 2004). CLEAs on the other hand are attractive because they can be made without extensive protein purification (Shah et al., 2006) and the final preparation has a high concentration of enzyme per unit volume (López-Serrano et al., 2002). Furthermore, cross-linking offers the advantages of improved enzyme activity, high enzyme stability, low production costs (owing to the exclusion of expensive carriers) and the possibility to co-immobilize different enzymes (Cao et al., 2003; Chen et al., 2006).

Cell-envelope proteinases (CEPs) are a special class of extracellular proteolytic enzymes obtained from lactic acid bacteria. In the food industry, CEPs improve the texture and organoleptic characteristics of dairy products and also have the potential to release bioactive peptides encrypted in dairy proteins (Tsakalidou et al., 1999; Agyei and Danquah, 2011). However, the use of the free CEPs in industrial processes is currently suboptimal and presents further drawbacks such as poor stability and lack of multiple utility. The most extensively used method of extracting CEPs is by washing or incubating the bacteria cells in a calcium-free buffer (Tsakalidou et al., 1999). However, this method compromises the final storage stability and thermal stability of the enzyme extract for some species (Hébert et al., 1997; Martín-Hernández et al., 1994). Additionally, Food and Agriculture Organization of United Nations (FAO) regulations suggest that enzyme preparations used in the production of consumables (such as food or pharmaceuticals) must be removed from products after processing (FAO/WHO, 2006) – a difficult if not impossible objective to achieve using soluble enzymes. The accumulative effect of all these factors makes soluble CEPs enzyme-based process economically unfeasible. CEPs expressed from *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) were selected for this study since their production and extraction have already been optimized in previous studies (Agyei et al., 2012; Agyei and Danquah, 2012b; Agyei et al., 2013). LDL 313 is an understudied lactobacilli species (Agyei et al., 2012). As such, screening for stable and recyclable biocatalysts from LDL 313 will make it economically feasible and enhance the application prospects of this species in the food industry. Further, to the best of our knowledge, no study has reported the preparation of CLEAs from lactobacilli CEPs. In this study therefore, cross-linked enzyme aggregate technology was used to develop stable forms of CEPs expressed by LDL 313. Conditions for quick and efficient CLEAs preparation were studied and the catalytic properties of aggregates were also tested on several food proteins.

2. Experimental

2.1. Strain and growth condition

Lactobacillus delbrueckii subsp. *lactis* 313 (ATCC® 7830™) was obtained from ATCC (Manassas, USA) and propagated twice in deMan, Rogosa and Sharpe (MRS) Broth (Acumedia, Michigan, USA) at 37 °C, 5% CO_2 and stored at −70 °C. The revived culture was grown to early stationary phase (optical density at 560 nm (OD_{560}) of ~1.0) in MRS broth. Culture was centrifuged and the cell pellets suspended in an equal volume of 50 mM phosphate buffer (pH 7). This was used to inoculate fresh MRS broth to an initial OD_{560} of 0.1. Anaerobic fermentation was carried out in 500 mL Erlenmeyer flasks, in a gyratoy shaker (100 rpm).

2.2. Preparation of crude cell-envelope proteinase

Culture was grown at 45 °C to stationary phase (optical density at 560 nm (OD_{560}) of ~2.8 ± 0.4, about 0.58 g/L dry cell weight), harvested by centrifugation (4000 × *g*, 10 min, 4 °C), and resuspended to an OD of 5 in the extraction solution containing 50 mM sodium phosphate buffer supplemented with 5 mM EDTA (pH 7) for cell-envelope proteinase (CEP) extraction via incubation (30 °C, 2 h, 50 rpm). The suspension was centrifuged (4000 × *g*, 10 min, 4 °C) and the supernatant was retained, filtered through 0.45 μm membrane filters (Acrodics, Pall Life Sciences) and designated as crude cell-envelope proteinase (CEP). Protein concentration was determined with a Protein Assay Kit according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

2.3. Preparation of cross-linked enzyme aggregates

The crude cell envelope proteinase (CEP) was placed in a micro centrifuge tube to which solid $(NH_4)_2SO_4$ was added to obtain a required level of w/v saturation. After brief vigorous mixing (~1 min), glutaraldehyde (25%, w/v in water) was added to give a desired final concentration. The mixture was incubated with shaking and CLEAs formed washed via centrifugation (10,000 × *g*, 5 min, and room temperature) until supernatant was colourless signifying removal of free glutaraldehyde. This required washing twice with buffer (50 mM Na-phosphate buffer, 1 M NaCl, pH 7) and a final rinse with fresh Na-phosphate buffer (50 mM, pH 7). The final enzyme preparation was kept in Na-phosphate buffer (50 mM, pH 7). If necessary, the solid was dispersed with the aid of a pipette tip. In some instances, unreacted carbonyl groups were quenched by reacting washed CLEAs with 1 M Tris–HCl pH 7.6 (250 rpm, 1 h, 20 °C) and washed twice with Na-phosphate buffer via centrifugation (10,000 × *g*, 5 min). For experiments on the effect of proteic feeders on CLEAs activity recovery, appropriate amounts (mg) of BSA from a 0.25 g/mL stock solution was added to crude CEPs before the addition of precipitation and cross-linking agents. Optimization of the cross-linking conditions (i.e. ammonium sulphate concentration, incubation temperatures, agitation speed, glutaraldehyde cross-linker concentration, $CaCl_2$ concentration, reaction time and the addition of proteic feeders) were done in a sequential manner and in the order presented by fixing values for all other factors and varying one. The results of the optimum value selected for one conditions is then used for the optimization of subsequent ones. A full description of the actual values which were fixed or varied is given for each condition in the figure caption in Section 3. The efficiency of immobilized enzymes was evaluated in terms of retained activity (%) as follows:

$$\text{Retained activity}(\%) = \frac{\text{Activity of immobilized enzyme aggregates}}{\text{Activity of free enzyme introduced}} \times 100\% \quad (1)$$

2.4. Proteinase activity assay

Proteinase assay was determined with the Protease Colorimetric Detection Kit (Sigma) with casein as substrate by measuring