Efficient preparation of silkworm pupae protein active peptide by protease displayed on the surface of *Bacillus subtilis*

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Bioactive peptides have been widely reported to be beneficial to the life activities of biological organisms, and their relative molecular mass is less than 6000 Da (Akbarian et al., 2022). Bioactive peptides have a variety of metabolic and physiological regulatory functions, such as easy digestion and absorption, promoting immunity, hormone regulation, antibacterial, antiviral, lower blood pressure, lower blood lipid and other effects (Tkaczewska et al., 2020). Because of its excellent biological functions, bioactive peptides have become the most popular and promising functional factors in the international food industry.

Enzymatic hydrolysis of protein is the most effective and green method for preparing bioactive peptides. However, the environmental sensitivity and cost of the enzyme limit the large-scale production of active peptides. In recent years, many studies have proved that it is feasible to improve enzyme yield and enzyme properties by modifying microorganisms to construct whole cell biocatalysts. Zhu et al. demonstrated carbonic anhydrase on the surface of *Escherichia coli* for CO_2 capture and mineralization, effectively improving the purification stability of carbonic anhydrase, and the problems of high cost, easy inactivation, unrecoverable biocatalyst and limited contact between substrate and enzyme are solved. (Zhu et al., 2023). Liao et al. used the surface display of yeast to immobilize LHyal directly and used it to hydrolyze high molecular weight hyaluronic acid to produce low molecular weight hyaluronic acid and hyaluronic acid oligosaccharides. The method avoids the complicated enzyme purification process and optimizes the purification of products such as hyaluronic acid oligosaccharides, which greatly reduces the production cost of high-value products (Liao et al., 2023). Therefore, the use of surface display to immobilize enzymes and apply to the preparation of high-value chemical products is a feasible scheme.

Surface display technology is a genetic engineering technology that connects heterologous peptides or proteins with anchor proteins in microbial cells, secretes and expresses them in the form of fusion proteins on the surface of host cells (Park et al., 2020), and can maintain the independent spatial conformation and biological activity of target peptides or proteins (Garcia-Galan et al., 2011). Studies have shown that, microbial surface display can give heteroproteins stronger tolerance and stability, higher binding efficiency to substrates, and easy recovery (Mei et al., 2017). In this study, alkaline protease was immobilized on the surface of *Bacillus subtilis*, and its schematic diagram is shown in Figure 1.

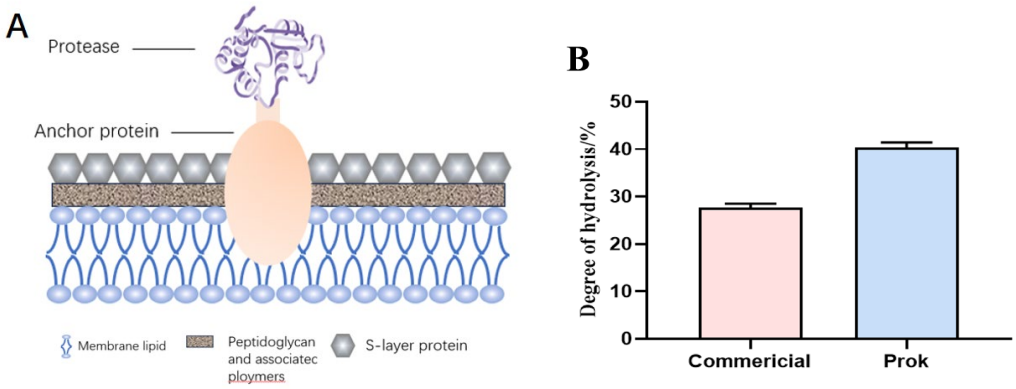

Figure 1. Schematic representation of protease ProK displayed on the surface of *Bacillus subtilis* (A), and the hydrolysis rate of silkworm pupae protein by protease Prok (B)

In this study, the surface display system of *Bacillus subtilis* was successfully constructed in the form of fusion protein. As shown in Figure 1A, Bsla as the anchor protein was fused with alkaline protease Prok. Subsequently, enzyme activity of alkaline protease Prok fixed on the cell surface was analyzed to hydrolyze silkworm pupae protein. Figure 1B shows the hydrolysis rate of silkworm pupae protein by protease Prok. The results showed that the hydrolysis rate of Prok protease was 39.2±3.5%, which was higher than that of common commercial enzymes. Therefore, using alkaline protease Prok to hydrolyze silkworm pupa protein can produce active peptide efficiently

Figure 2 shows some basic properties of alkaline protease Prok, such as temperature stability, pH stability and so on. The results showed that alkaline protease Prok has good stability in a wide range of pH. Under the condition of pH 9, enzyme activity of Prok is the highest; In the optimization of temperature conditions, enzyme activity of Prok is the highest at the temperature of 60℃. Next, Prok will be used for enzymatic hydrolysis of silkworm pupae protein at pH 9 and temperature 60℃, so as to efficiently prepare bioactive peptides.

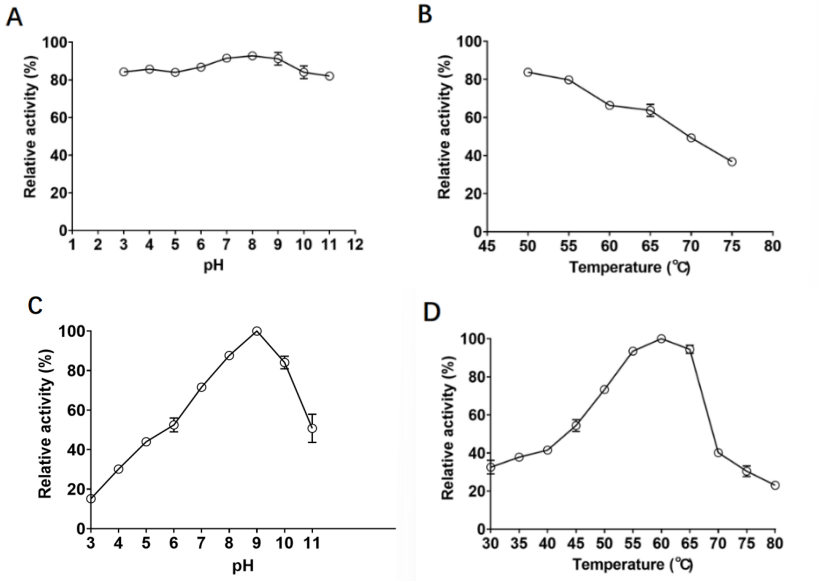

Figure 2. Enzymatic properties of protease Prok

(A) Optimum temperature; (B) Optimum pH (C) Temperature stability; (D) pH stability;

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