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Antibacterial activity of protease hydrolysates isolated from *Silybum marianum*

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In this study, the antibacterial activity of protease hydrolysates from *Silybum marianum* protein isolates (SMPIs) was investigated. Neutral protease, papain, pepsin and alkaline protease were used as experimental enzymes, while *Escherichia coli*, *Staphylococcus aureus*, *Sarcina lutea* and *Bacillus subtilis* were the bacterial indicators. The results showed that neutral protease, papain and pepsin hydrolysates exerted inhibitory effects on the four types of bacteria tested. However, alkaline protease hydrolysates of SMPI showed stimulatory effects on replication of the four bacteria tested. The antibacterial mechanism of SMPI hydrolysates was studied using scanning electron microscopy, and the results showed effective inhibition of *E. coli* (Gram-negative, G^-) and *S. aureus* (Gram-positive, G^+). It is speculated that the underlying mechanism of SMPI hydrolysates may involve injury to *E. coli* and *S. aureus* cell membranes. Currently, no similar studies have been conducted on the antibacterial activity of SMPI.

Keywords: Antibacterial activity, antibacterial mechanism, proteolysis, *Silybum marianum*.

SILYBUM MARIANUM (L.) Gaertn, also known as milk thistle, is an annual or biennial herbaceous plant belonging to the composite family. Originally from the Mediterranean, it is now distributed worldwide[1,2]. From ancient times, *S. marianum* has been considered a folkloric hepatoprotective herb. Its constituents include silymarin, milk thistle oil[3] and protein that are known to have high medicinal value[4,5].

Literature reports specify that *S. marianum* is mainly used for extraction of silymarin[6] for clinical and pharmaceutical applications[7–9] while a large portion of its protein content remains in the spent meal, which is used as forage or simply discarded after the extraction process. At the end of the last century, Chen, Wang[10] and Zhu[11] studied *S. marianum* protein and confirmed that it contains all the amino acids, and suggested it to be a potential

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plant protein source. In recent years, the greatly increased global demand for *S. marianum* has caused researchers to intensify their studies on *S. marianum*. Research on *S. marianum* protein has mainly focused on extraction methods[12], certain properties and preparation of antioxidant peptides by enzymatic hydrolysis[13,14]. There are currently no relevant studies in the literature on the antibacterial activity of *S. marianum* protein. Therefore, this study is aimed at performing enzymolysis of *S. marianum* protein isolates and evaluating the antibacterial activity of the enzymatic hydrolysate. Furthermore, the study is also aimed to elucidate the properties and underlying mechanisms of this potential new source of plant proteins and, thereby, provide a scientific base for future applications.

S. marianum seeds were provided by TianYuan Pharmaceutical Inc., (Panjin, Liaoning, China). Neutral protease, papain, pepsin and alkaline protease with enzyme activities of 200,000, 800,000, 3,000 and 200,000 U/g respectively, were purchased from Ruiyang Biotechnology (Wuxi, Jiangsu, China). *Escherichia coli*, *Staphylococcus aureus*, *Sarcina lutea* and *Bacillus subtilis* were provided by the Microbiological Laboratory of the Shenyang University of Chemical Technology. All other chemicals used were of analytical grade and purchased from the Shenyang Guoyao Group (Shenyang, Liaoning, China).

S. marianum seeds were powdered and degreased for 24 h using a moderate amount of ether and filtered through an 80-mesh sieve. A moderate amount of degreased seed powder was then added to water at a powder : water ratio of 1 : 16. The pH was adjusted to 11 using 0.5 mol/l sodium hydroxide (NaOH) solution and the mixture was then placed in a 50°C water bath for 1 h and centrifuged for 20 min at 4000 rpm, and the supernatant obtained. The pH of the supernatant was adjusted to 5.5 with hydrochloric acid (HCl) followed by centrifugation for 20 min at 4000 rpm. The precipitate was then collected and lyophilized by freeze-drying, and the resulting powder was the *S. marianum* protein isolate (SMPI).

Four enzymes including neutral protease, papain, pepsin and alkaline protease were used as experimental enzymes in this study. Sterile water was added to SMPI at an 80 : 1 ratio (w/w). After mixing thoroughly, the pH of the mixture was adjusted to the optimum value for all the proteases. Following a 10-min incubation at optimum temperature for each enzyme and based on the mass of SMPI, 2000 U of protease was added for every gram of SMPI for enzymolysis, with the pH being maintained at the optimum range for each protease. After a 5 h enzymolysis, the mixture was heated to 95–100°C for 10 min to deactivate the enzyme, and the pH was neutralized. Following centrifugation at 7000 rpm for 20 min at 4°C, the upper oil layer and the residue at the bottom of the mixture were discarded and the middle supernatant layer was stored at –20°C for future use.

E. coli, *S. aureus*, *S. lutea* and *B. subtilis* were used as the bacterial indicators under optimum enzymatic hydrolysis conditions for each enzyme, and the inhibitory effects of all enzymatic hydrolysates were determined against the bacterial indicators. Experiments were conducted according to methods previously described by Dionysius[15] and Song[16] with modifications. A 1 ml sample of the SMPI enzymatic hydrolysate and 100 µl of the bacteria indicator suspension (10^7 cfu/ml) were added to 5 ml of beef extract peptone liquid medium followed by mixing and culturing at 37°C for 24 h. The blanks consisted of 5 ml beef extract peptone liquid medium and 1 ml sterile saline. The absorbance of the samples and controls was measured at 600 nm and the final values calculated using the following formula

$$\text{Antibacterial rate (\%)} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100,$$

where A_{blank} and A_{control} are the absorbance values of the culture medium and the sterilized saline blanks with other conditions maintained. Each sample was run in quadruplicate, and the values were used to calculate the bacterial inhibition rate of the SMPI enzymatic hydrolysate.

The underlying mechanism of the antibacterial peptides may be mediated by cell membrane or non-membrane damages. Membrane damaging peptides inhibit bacteria by destroying their cellular membrane structure, while non-membrane damaging peptides bypass the cell membrane and bind to DNA, RNA, and cell proteins to inhibit the biosynthesis of these molecules[16]. Based on the results obtained from scanning electron microscopy (SEM), hypotheses of the possible antibacterial mechanisms of the SMPI enzymatic hydrolysates were proposed and conducted relevant experiments to confirm them were conducted; the details are as follows.

The typical Gram-negative bacterium – *E. coli* (G^-) and Gram-positive bacterium – *S. aureus* (G^+) were chosen as the experimental bacteria. The pepsin hydrolysate from SMPI was mixed with 1 ml of a suspension of the two bacteria (10^7 cfu/ml) while the bacterial suspension was substituted with the same volume of sterile water for the control sample. The mixtures were cultured at 37°C for 18 h, centrifuged at 4°C (3000 rpm, 10 min) to discard the supernatant, washed with sterile saline solution, and then further centrifuged twice. The samples were then fixed with 2.5% glutaraldehyde at 25°C for 12 h and washed thrice with 0.1 mol/l phosphate buffer (pH 7.2) under the same conditions as above. Subsequently, the samples were sequentially dehydrated in 30%, 50%, 70%, 90%, and 100% ethanol and finally air-dried to obtain the anhydrous samples. The samples were observed under the scanning electron microscope after vacuum gold plating.

As shown in Figure 1, the different protease hydrolysates of SMPI induced differing degrees of antibacterial

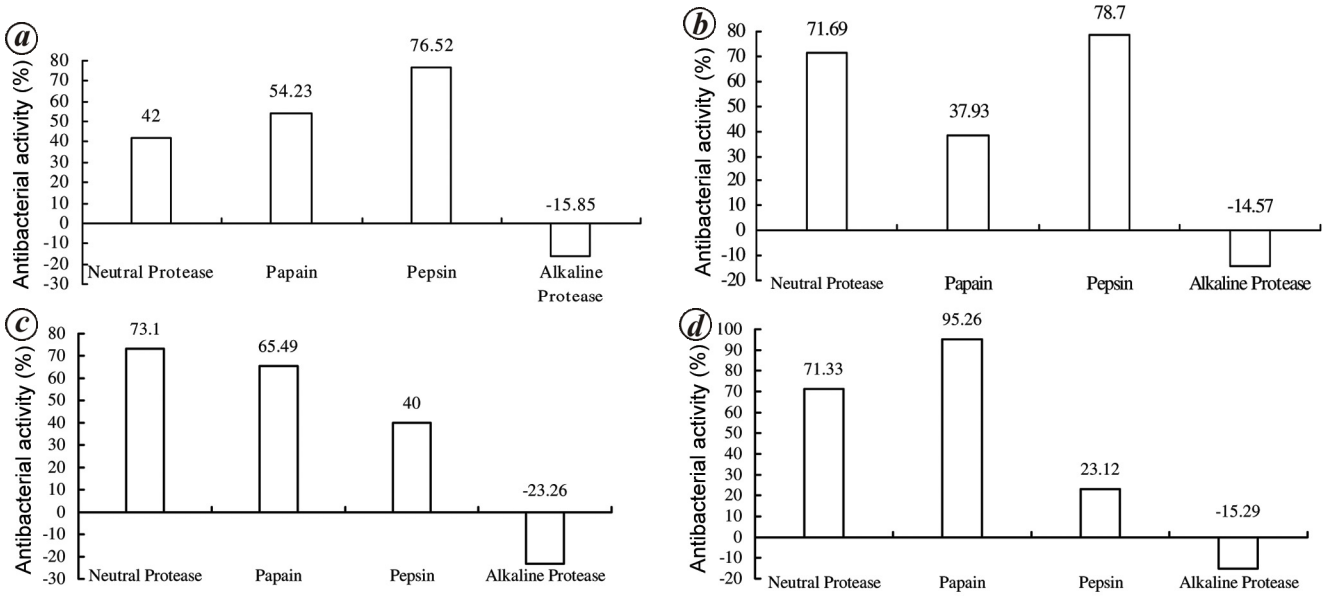

Figure 1. Inhibition of bacteria by enzymatic protein hydrolysates. Comparison of rate of inhibition of four bacteria tested by *Silybum marianum* protein isolates enzymatic hydrolysates.

activity against the four bacteria tested. The *E. coli* (G^-) group (Figure 1 *a*) showed that SMPI pepsin hydrolysate showed the strongest inhibitory effect against *E. coli* (76.52%) followed by the hydrolysates of papain and neutral protease (54.23% and 42.00% respectively). However, alkaline protease hydrolysate exhibited a stimulatory effect on the replication of *E. coli*, which was determined to be –15.85%.

The trends were similar against *S. aureus* (G^+), with the SMPI pepsin hydrolysate showing the strongest inhibitory effect, followed by that of the hydrolysates of neutral protease and papain (Figure 1 *b*). Further, similar to its effects on *E. coli*, the alkaline protease hydrolysate exhibited a stimulatory effect on the growth of *S. aureus*, measured as –14.57%.

Figure 1 *c* shows that the SMPI neutral protease hydrolysate showed the highest inhibition of *S. lutea* (G^+), followed by hydrolysates of papain and pepsin. The alkaline protease hydrolysate stimulated the replication of *S. lutea*, measured as –23.26%.

The different protease hydrolysates of SMPI induced differing degrees of antibacterial activity against the *B. subtilis* (G^+, with spores) as well (Figure 1 *d*). The papain hydrolysate showed the highest activity against *B. subtilis*, which was followed by the activities of neutral protease and papain hydrolysates in the given order. However, alkaline protease hydrolysate stimulated the growth of *Bacillus subtilis*, measured as –15.29%.

We used SEM to visualize the morphological changes in the bacterial cell membrane induced by the SMPI enzymatic hydrolysates. The effects on *E. coli* and *S. aureus* are shown in Figure 2. The control group for *E. coli* (Figure 2 *a*) and *S. aureus* (Figure 2 *c*) showed morphological characteristics of normal *E. coli* and *S. aureus* including a short rod-shaped morphology with a smooth and intact surface, and grape-like clusters, with a smooth and intact surface with no visible damage to the cell membrane respectively. Significant changes were observed in the morphological structure of both *E. coli* and *S. aureus* after treatment with the SMPI pepsin hydrolysate – indentations of the bacterial cell membrane, which formed visible holes, were evident (Figure 2 *b* and *d*), and some cell membranes were completely destroyed and visibly disintegrated. According to the classification criteria for antibacterial peptides, the mechanism by which the SMPI enzymatic hydrolysate inhibits *E. coli* and *S. aureus* growth can be classified as inhibition by membrane damage.

Therefore, based on the results, it was speculated that the antibacterial mechanisms of SMPI enzymatic hydrolysates against G^- and G^+ bacteria should both be classified as inhibition via membrane damage.

The structure and functionality of proteins can both change after protease enzymolysis, which can produce peptide fragments with unique properties and features. The peptides differ from original proteins and have a relatively small molecular mass. In addition, they have numerous hydrolysis-induced structural rearrangements, which expose the hydrophobic sites that were originally embedded inside the protein to solvent. Therefore, enzymatic hydrolysis of proteins can serve as an important strategy for further isolating and producing biologically active compounds[17].

The SMPI neutral protease, papain and pepsin hydrolysates exhibited antibacterial effects against *E. coli*, *S. aureus*, *S. lutea* and *B. subtilis*, while the alkaline protease

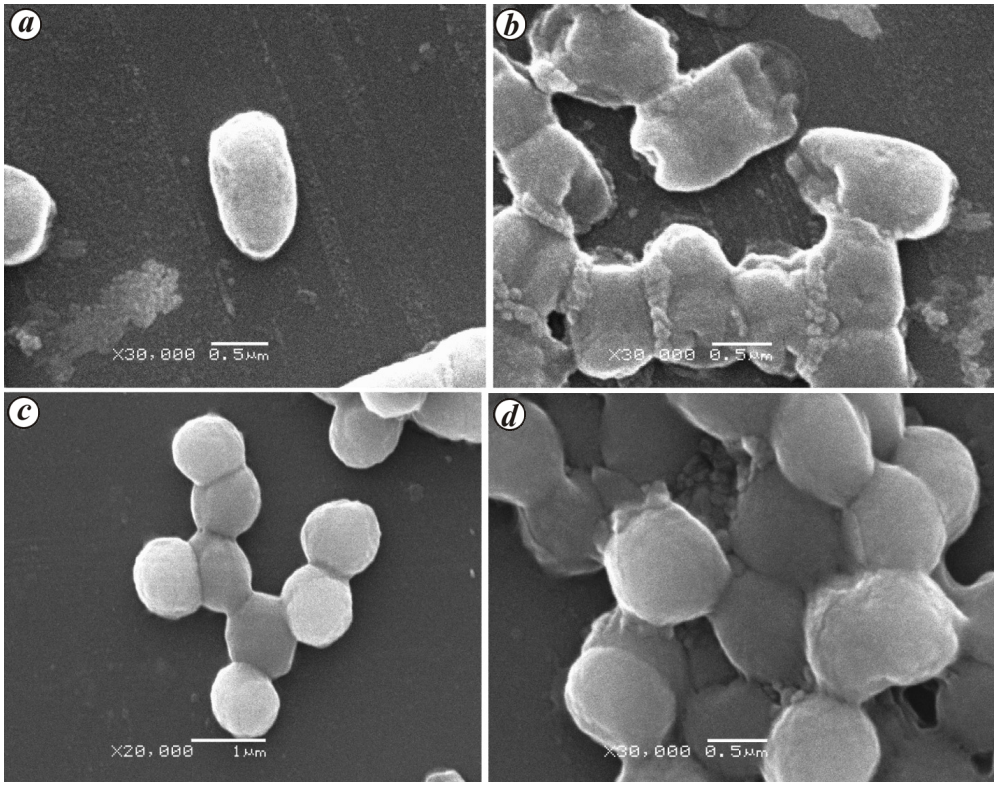

Figure 2. Scanning electron microscopy of inhibition of bacteria in control and *Silybum marianum* protein isolates hydrolysate-treated groups. ***a***, *E. coli* control; ***b***, SMPI enzymatic hydrolysate groups; ***c***, *S. aureus* control; ***d***, SMPI enzymatic hydrolysate groups.

hydrolysate promoted replication of these four bacteria. Furthermore, neutral protease, papain and pepsin are highly specific endonucleases that can release antibacterial fragments hidden inside the SMPI. The main restriction sites of neutral protease and pepsin are surrounded by hydrophobic (or nonpolar) amino acids such as phenylalanine (Phe) and leucine (Leu)[18]. Within a certain range, more hydrophobic residue fragments in enzymatic hydrolysate enhance the favourable combination with lipophilic proteins on the cell membrane, which subsequently mediates inhibitory effects via specific antibacterial mechanisms. Papain mainly cleaves the peptide bond formed by the carboxyl groups of lysine (Lys), arginine (Arg) and Phe[19]. Although there are not many hydrophobic residue fragments in enzymatic hydrolysate, it contained a high concentration of cations and hydrogen bonds, which are unique to arginine containing peptides. These elements favour their binding to numerous anionic components on the bacterial cell membrane, which mediates their antibacterial effects by formation of analogous structures[20].

The stimulatory effects of alkaline protease hydrolysate on the replication of bacterial indicators may be related to the characteristics of alkaline proteases, which are serine proteases that mainly cleave the carboxyl groups of hydrophobic amino acids. Following the hydrolysis of SMPI by alkaline proteases, numerous hydrophobic residues become exposed. The high hydrophobicity results in coagulation and precipitation of peptides, therefore rendering the enzymatic hydrolysate void of any antibacterial activities. Therefore, the remaining polypeptide and other biological compounds in the solution after enzymolysis would serve as additional sources of nitrogen for growth of bacteria, and subsequently promote their replication.

Conflict of interest: The authors declare that they have no conflict of interest.

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Isolation and characterization of phosphorus solubilizing bacteria from manganese mining area of Balaghat and Chhindwara

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Plants require optimum amount of available phosphorus to support their growth and development. Phosphorus is known to have significant role in root subdivision, vitality and disease resistance of plants. Different types of bacteria involved in phosphorus solubilization can be used as biofertilizer in reclamation of mining area. The present study deals with isolation and identification of phosphorus solubilizing bacteria from the manganese mining area of Balaghat and Chhindwara districts of Madhya Pradesh, India. rDNA (16s) based molecular identification was performed assisted by MEGA phylogenetic analysis. *Pseudomonas putida*, *Bacillus licheniformis*, *Pseudomonas taiwanensis* and *Pseudomonas aeruginosa* were explored as potential phosphorus solubilizers from the selected sites.

Keywords: Mining area, phosphorus solubilizing bacteria, 16s rDNA.

PHOSPHORUS is one of the essential elements in soil but present in very low concentration in available form. Iron, calcium and aluminium rapidly immobilize inorganic available phosphorus and convert it into unavailable format of tricalcium phosphate, iron phosphate and aluminium phosphate. About 20% of soil phosphorus occurs in organic form and the general concentration of available phosphorus exists about 2 μM and rarely exceeds 10 μM (ref. 1). But plant tissues require a minimum of 5–20 mM of available phosphorus for rich growth and development[2,3]. To increase the available phosphorus of soil certain chemical fertilizers with inorganic phosphorus need to be added, but this affects the soil quality[4]. Numerous microbes, specially bacteria, are potential solubilizers of phosphorus and used as biofertilizers in agricultural lands. The role of microorganisms in solubilization of soil attached or soil precipitated phosphorus is already a focused area in recent studies. Most of the phosphate solubilizing bacteria (PSB) belong to genera: *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Bacillus*, *Burkhoderia*, *Azotobacter*, *Azospirillum*, *Mesorhizobium* and *Erwinia*[5–9]. The general mechanism of phosphorus solubilization

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