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New Material: Translucent Balsa-P-Coumaric Acid Modified Lysozyme Dressing

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Abstract. objective: using p-coumaric acid as modifier, dopamine adhesive prepared modified lysozyme, using bacteriostatic circle diameter, bacteria culture and the minimum bacteriostasis concentration bacteriostatic function, the optimal PH, temperature, time, contrast enzymology properties, and hydrophobicity and secondary structure content determination. Methods: the preparation of translucent Balsa, respectively designed for Control group (Control), wood group (A), translucent Balsa group (B), translucent Balsa - lysozyme group (C), translucent Balsa - P-coumaric acid modified lysozyme group (D). Results :(1) the bacteriostatic effect and the minimum inhibitory concentration of the P-coumaric acid-modified lysozyme > P-coumaric acid; The surface hydrophobicity index of the three substances and the stability of the secondary structure were all p-coumaric acid - modified lysozyme > lysozyme > P-coumaric acid; PH = 6, the temperature is 45°C, the response time of 30 min strongest P-coumaric acid modified lysozyme activity; (2) the Drug loading and encapsulation rate of group D were better than that of group C. In terms of the release curve, the release of C and D groups in the 48h group tended to be stable and maximum, and the cumulative release percentage of 72h was 85.2% and 93.8% respectively. (3) in the control group, the growth of staphylococcus aureus and e. coli was basically in line with the normal trend, and the antibacterial activity of e. coli and staphylococcus aureus in each group was D>C>B ($P<0.05$). And 1-7 days, different groups had no value inhibition on fibroblasts ($P>0.05$); Conclusion: This study successfully optimized the design of modified lysozyme to prepare Translucent Balsa-P-coumaric acid Modified, which has strong antibacterial ability, stable and persistent release, and no cytotoxicity.

Key words: Balsa; Lysozyme; P-coumaric acid; dopamine.

INTRODUCTION

Chemical modification of enzyme molecules refers to change certain properties of enzymes for the purpose, by cutting and splicing of main chain side chain of chemical modification, modification of enzyme molecules, thus creating natural enzyme molecules without some excellent characters to enlarge the application range of enzyme, so as to achieve a higher economic efficiency[1-2]. In recent years, some researchers through the experiment, carried out on the side chain groups of natural lysozyme chemical modification, form an enzyme that amphiphilic molecules, making modification of enzyme molecules by gram-negative bacteria lipopolysaccharide layer, thereby gaining the sterilization ability of gram-negative bacteria[3-4]. Therefore, the main purpose of this study is how to obtain the low cost, high recovery of high quality products of lysozyme, and for its inhibition of gram-negative bacteria weaker this defect, through modification research, in order to obtain the gram-negative bacteria have the modified enzyme inhibitor activity[5].

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MATERIALS AND METHODS

Material Preparation

Preparation of P-Coumaric Acid Modified Lysozyme

It is accurate to say that 60 mg of P-coumaric acid is dissolved in 3mL5M NaOH, and the pH of 5M is adjusted to 7.5, and the sterilization deionized water to 8 mL is added, and 160 mg of dopamine is added, and the room temperature is still 1h after completely dissolved. Add 60 mg lysozyme, 30°C constant temperature water bath stirring for 24 h, the reaction after the insoluble part of centrifugal (6000 r/min, 20 min), soluble part on Sephadex G - 25 column, collected with enzyme activity of elution peak, dialysis 1 d under 4°C, enrichment, save after freeze drying.

Preparation of Semi-Transparent Balsa - P-Coumaric Acid Modified Lysozyme

It was said that 131.14mgTris hydrochloric acid was dissolved in 100ml deionized ions, and then the dopaminergic powder was added to 200mg, which was prepared to be 2mg/ml with a PH of 8.5 Tris- dopaminergic solution. Then will Balsa soaking solution with dopamine in 12 h, placed in 37°C table, 100 r/min. Each of them was dissolved in 50ml deionized water by 500mg lysozyme and p-coumaric acid - modified lysozyme powders. The solution was prepared in 10mg/ml of lysozyme final concentration solution, which was divided into C and D groups respectively. Translucent Balsa with deionized water to clean, the dip into in C, D, and two groups of 12 h in the solution, placed in 37°C table, 100 r/min, out after the reaction at room temperature aseptic saved for later use.

Preparation of Semi-Transparent Balsa.

Select the Balsa with a density of about 160 kg/m3, perforate with punch, and make a round piece with a diameter of 0.6mm and a thickness of 0.8mm. In the 105±3°C under the environment of dry 24 hours, and then soaked in 1% concentration of NaClO2 - acetate buffer (80°C) PH4.6, 12 h of reaction. After careful cleaning, the sample was removed by using deionized water, followed by ethanol - 1:1 ethanol mixed with acetone - acetone in three steps, 10min each step, and repeated three times. Then infiltration lignin, namely methacrylate (MMA) polymerization formaldehyde solution, heating the oven to 70°C for 4 h. Finally, the infiltrating template is sandwiched between two pieces of glass and is further polymerized with aluminum foil. The polymerization process by heating the oven to 70°C 4 h wood finish.

Optimization and Functional Evaluation of Modified Lysozyme

Determination of Bacteriostatic Ring

It was suggested that a certain amount of lysozyme and p-coumaric acid modified lysozyme were dissolved with 50 mM pH 7.0 phosphate buffer solution, and a sample solution of 20 mg/mL was accurately prepared. The cups and saucers method is adopted to gram positive bacteria (staphylococcus aureus) and gram negative bacteria (e. coli) in vitro bacteriostasis experiment, take 20 mL melting has sterilization of solid cultivation based on petri dishes, let stand after being frozen, take 0.2 mL diluted good concentration for 106 ~ 107 cfu/mL bacteria suspension uniformly coated on the tablet, after 10 min, placed the Oxford cup in a petri dish on the surface, to join different samples of the fluid, cover petri dishes, 37°C constant temperature culture 24 h, bacteriostatic circle diameter of the petri dish. Each sample solution was repeated three times and averaged.

Bacterial Co-Culture

Staphylococcus aureus (S.aureus) and Escherichia coli (e. coli) strain amplified bacteria (the bacteria to sleep in the night) to 1*109CFU/ml, and the diluted bacterial solution was 1*104CFU/ml in LB medium, and the OD value was detected by 100ml of the enzyme standard. The OD value was about 0.07, which was the standard. Take two 96 - well plates, snapping the lysozyme and modified enzyme placed in the hole, after each group of three holes, each hole to join 200 ul mixed bacteria liquid, 37°C table 12 h after incubation test each OD value change, repeat three times.

Determination of Optimal Ph, Temperature and Time

Accurately according to a certain amount of lysozyme and p-coumaric acid modified lysozyme, soluble in 50 mM different pH (4.0, 5.0, 6.0, 5.0, 6.0) in the phosphate buffer, precise mixture concentration for 2 mg/mL solution, respectively in different temperature (45°C, 50°C and 40°C and 55°C, 60°C) different constant temperature water bath reaction time (3 min, 15 min, 30 min, 1 h, 2 h). Rapid cooling to room temperature, the determination of lysozyme and P-coumaric acid modified lysozyme in different Settings under the condition of enzyme activity, for the convenience of compare the determination of a variable changes, among other variables constant value, as one of the highest enzyme activity was 100%, under the condition of other relative enzyme activity of the enzyme activity and the percentage of the highest enzyme activity.

Hydrophobicity Determination

Accurately according to a certain amount of lysozyme and p-coumaric acid modified lysozyme, soluble in 10 mM respectively in phosphate buffer pH 7.0, precision dispensing concentration gradient is 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL of the sample fluid, fluid samples from different concentration gradient respectively 4 mL, add 50 mu L ANS solution (with 10 mM pH7.0 mixture of 8 mM phosphate buffer solution), excitation wavelength of 360 nm, emission wavelength of 470 nm, fluorescence intensity was determined by fluorescence.

Determination of Secondary Structure

The solid sample was mixed with KBr, grinding and pressing, and Nicolet iS10 Fourier transform infrared spectrometer was selected for infrared spectral scanning. Experimental conditions: spectral resolution 4 cm-1, wave number scanning range 4000 ~ 400 cm-1, scanning number 32.

Drug Loading,Encapsulation Efficiency and Drug Release In Vitro

Drug loading = actual concentration * volume (ug)/dissolved Balsa - lysozyme or modified enzyme total mass (mg);

Encapsulation efficiency = actual concentration/theoretical concentration.

RESULTS

Determination of Bacteriostatic Ring, Co-Culture and Minimum Inhibitory Concentration of Bacteria

PH = 6, the temperature is 45°C, the response time of 30 min strongest P-coumaric acid modified lysozyme activity (figure 1).The surface hydrophobicity index of the three substances and the stability of the secondary structure were all p-coumaric acid - modified lysozyme > lysozyme > P-coumaric acid (figure 2). The bacteriostatic effect and the minimum inhibitory concentration were the P-coumaric acid - modified lysozyme > lysozyme > P-coumaric acid (figure 3).

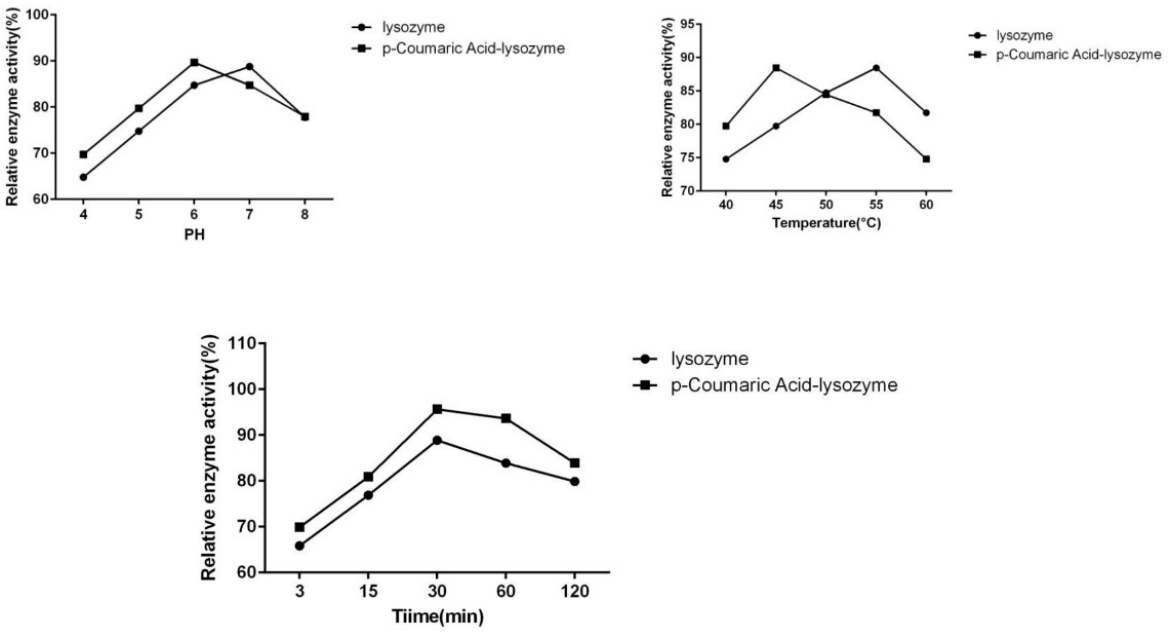
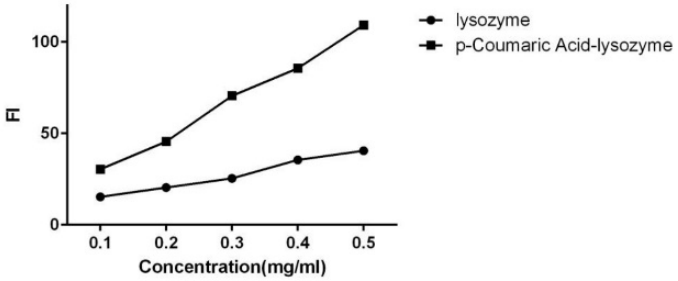
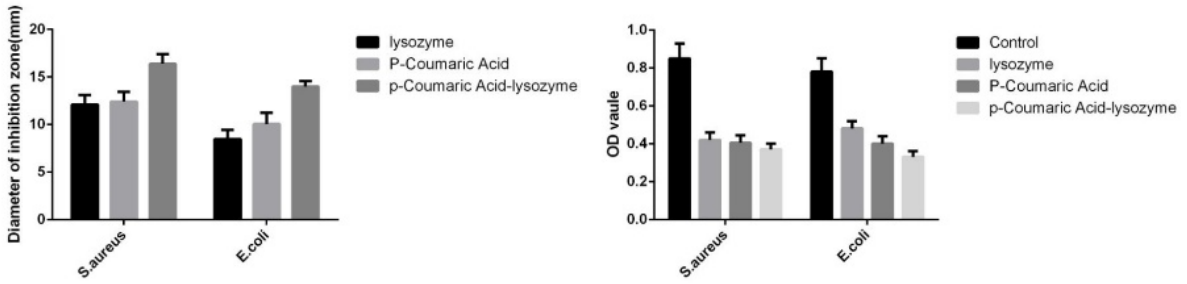


FIGURE 1. The optimal temperature, PH and reaction time of lysozyme and modified lysozyme.



Sample	α-helix	β-sheet	β-turn	Random coil
lysozyme	28.79	22.38	20.18	28.65
p-Coumaric Acid-lysozyme	26.54	24.19	23.92	25.35

FIGURE 2. The hydrolysis of lysozyme and modified lysozyme and the comparison of secondary structure.



Strain	Sample	2mg/ml	1.5mg/ml	1mg/ml	0.5mg/ml	0.25mg/ml	Control
S.aureus	lysozyme	-	-	-	+	++	++
	P-Coumaric Acid	-	-	+-	+	++	++
	p-Coumaric Acid-lysozyme	-	-	-	+-	+	++
E.coli	lysozyme	-	+-	+	+	++	++
	P-Coumaric Acid	-	-	+-	+	++	++
	p-Coumaric Acid-lysozyme	-	-	-	+-	+	++

FIGURE 3. Comparison of bacteriostatic effect and minimal inhibitory concentration of lysozyme, p-coumaric acid and modified lysozyme.

Drug Loading, Encapsulation Rate and in Vitro Drug Release Research

The Drug loading and encapsulation rate of group D were better than that of group C. From the release curve, the release of C and D groups in the 48h group tended to be stable and maximum, and the cumulative release percentage of 72h was 85.2% and 93.8% (Table 1).

TABLE 1. Drug loading and encapsulation efficiency in different groups

	C	D
Drug loading (μg/mg)	43.6±6.2	48.2±4.1
Encapsulation efficiency (%)	72.1±6.9	78.8±7.0

In Vitro Antibacterial and Cell Proliferation Inhibition

In the control group, the growth of staphylococcus aureus and e. coli was basically consistent with the normal trend, and the antibacterial activity of e. coli and staphylococcus aureus in each group was D>C>B ($P<0.05$). And 1-7 days, different groups had no value inhibition on fibroblasts ($P>0.05$) (Table 2-3).

TABLE 2. OD values of co-culture of different concentrations of S.aureus. and E.coli for 24h

	Control	A	B	C	D
S.aureus.	0.896±0.079	0.846±0.071	0.826±0.081	0.457±0.039	0.308±0.031
E.coli.	0.738±0.071	0.718±0.069	0.708±0.065	0.327±0.033	0.241±0.025

TABLE 3. Cytotoxicity's of different concentrations of dressings on the mice fibroblasts.

	1Day	3Day	5Day	7Day
Control	0.193±0.018	0.253±0.026	0.380±0.037	0.529±0.053
A	0.187±0.020	0.235±0.025	0.349±0.036	0.492±0.050
B	0.173±0.018	0.221±0.028	0.338±0.035	0.478±0.049
C	0.177±0.019	0.219±0.029	0.343±0.037	0.498±0.052
D	0.173±0.017	0.234±0.027	0.324±0.038	0.454±0.048

DISCUSSION

PH = 6, the temperature is 50°C, the response time of 30 min strongest P-coumaric acid modified lysozyme activity (figure 1). We believe that changes occur in the most appropriate pH of the chemically modified lysozyme due possibly to the fact that the covalent binding of different acids by natural enzymes leads to changes in isoelectric point as well as the enzyme structure[6-7]. Three kinds of enzyme surface hydrophobicity of the index of p-coumaric acid modified lysozyme > lysozyme > p-coumaric acid (figure 2), probably because after the modification of lysozyme, some changes have taken place in structure [8-9]. As shown in figure 3, bacteriostatic effects, and the minimum bacteriostasis concentration for p-coumaric acid - modified lysozyme > lysozyme > P-coumaric acid. This may be due to the fact that the modified lysozymes experience some changes in their structure thus exposing the hydrophobic groups within the natural enzymes and increasing the surface hydrophobicity index of natural enzymes[10].

In this study, The Drug loading and encapsulation rate of group D were better than that of group C. From the release curve, the release of C and D groups in the 48h group tended to be stable and maximum, and the cumulative release percentage of 72h was 85.2% and 93.8% (Table 1). This may be due to the fact that the p-coumaric acid-modified lysozyme is more likely to cover the balsa surface during preparation of pellets. In the control group, the growth of staphylococcus aureus and e. coli was basically consistent with the normal trend, and the antibacterial activity of e. coli and staphylococcus aureus in each group was D>C>B ($P<0.05$). And 1-7 days, different groups had no value inhibition on fibroblasts ($P>0.05$) (Table 2-3). This may be due to the fact that the lysozyme originates from egg white and is a natural and safe enzyme. It is believed that the safety of lysozyme allws for its wide application in food preservation [11-12].

To sum up, this study successfully optimized the design of modified lysozyme, prepared semi-transparent and Balsa - modified lysozyme dressing, which has strong antibacterial ability, stable and persistent release, and no cytotoxicity.

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