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Balsa-Cinnamic acid Modified Lysozyme Dressing

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Abstract. The research aims to using cinnamic 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. the bacteriostatic effect and the minimum inhibitory concentration of the Cinnamic acid-modified lysozyme > Cinnamic acid; The surface hydrophobicity index of the three substances and the stability of the secondary structure were all cinnamic acid - modified lysozyme > lysozyme > Cinnamic acid; PH = 6, the temperature is 50°C, the response time of 30 min strongest Cinnamic acid modified lysozyme activity; 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 92.0% respectively. 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 ($P>0.05$).

Key words: Balsa; Lysozyme; Cinnamic acid; dopamine.

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INTRODUCTION

Lysozyme has many incomparable advantages by chemical preservatives, and more and more be taken seriously, has wide application prospect, the research of this paper for the preparation and modification of egg white lysozyme, bacteriostasis, enzymology properties and structure to provide certain theoretical basis[1-2]. This study on the modified with chemical method, by the related reports, the modification of the enzymes is not single, but a mixture of different modification degree of modification of enzyme, the follow-up study of this study are to the mixture, can further to isolate the components in the mixture, further study on the properties of each single component[3-4]. And in terms of the structure of the enzyme. This study measured the modification enzyme mixture of different secondary structure, the changes of enzymes play a role of main part is the active center, can further research before and after the modification of enzyme activity changes of the center, for the study of the enzymatic properties of the basis of system theory[5-6].

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MATERIALS AND METHODS

Material Preparation

Preparation of Cinnamic Acid Modified Lysozyme

It is accurate to say that 60 mg of Cinnamic 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 - Cinnamic 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 cinnamic 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.

Optimization and Functional Evaluation of Modified Lysozyme

Determination of Bacteriostatic Ring

It was suggested that a certain amount of lysozyme and cinnamic 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 cinnamic 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 Cinnamic 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.

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Hydrophobicity Determination

Accurately according to a certain amount of lysozyme and cinnamic 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.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). T-tests and one-way ANOVA were used to evaluate statistical significance, followed by post-hoc least significant difference tests. Values of $p<0.05$ were considered significant.

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RESULTS

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

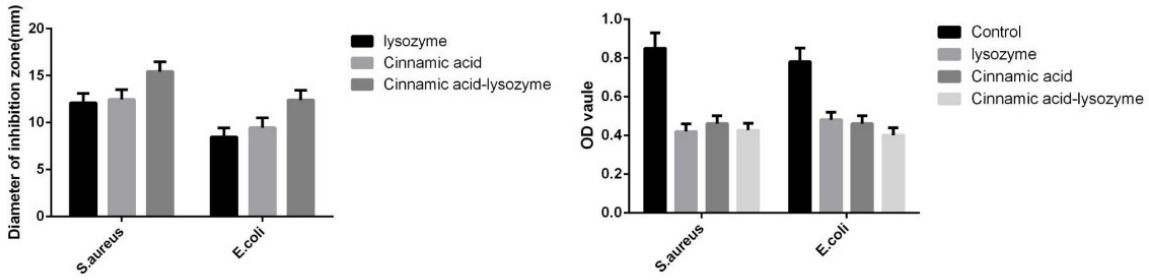
PH = 6, the temperature is 50°C, the response time of 30 min strongest Cinnamic acid modified lysozyme activity (figure 1).The surface hydrophobicity index of the three substances and the stability of the secondary structure were all cinnamic acid - modified lysozyme > lysozyme > Cinnamic acid (figure 2). The bacteriostatic effect and the minimum inhibitory concentration were the Cinnamic acid - modified lysozyme > lysozyme > Cinnamic acid (figure 3).

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 92.0% (Table 1).

TABLE 1. Drug loading and encapsulation efficiency in different groups

	C	D
Drug loadingn (μg/mg)	43.6±6.2	45.7±4.7
Encapsulation efficiency (%)	72.1±6.9	75.4±6.3



Strain	Sample	2mg/ml	1.5mg/ml	1mg/ml	0.5mg/ml	0.25mg/ml	Control
S.aureus	lysozyme	-	-	-	+	++	++
	Cinnamic acid	-	-	+-	+	++	++
	Cinnamic acid-lysozyme	-	-	-	+	++	++
E.coli	lysozyme	-	+-	+	+	++	++
	Cinnamic acid	-	-	+-	+	++	++
	Cinnamic acid-lysozyme	-	-	-	+	++	++

FIGURE 1. Comparison of bacteriostatic effect and minimal inhibitory concentration of lysozyme, cinnamic acid and modified lysozyme.

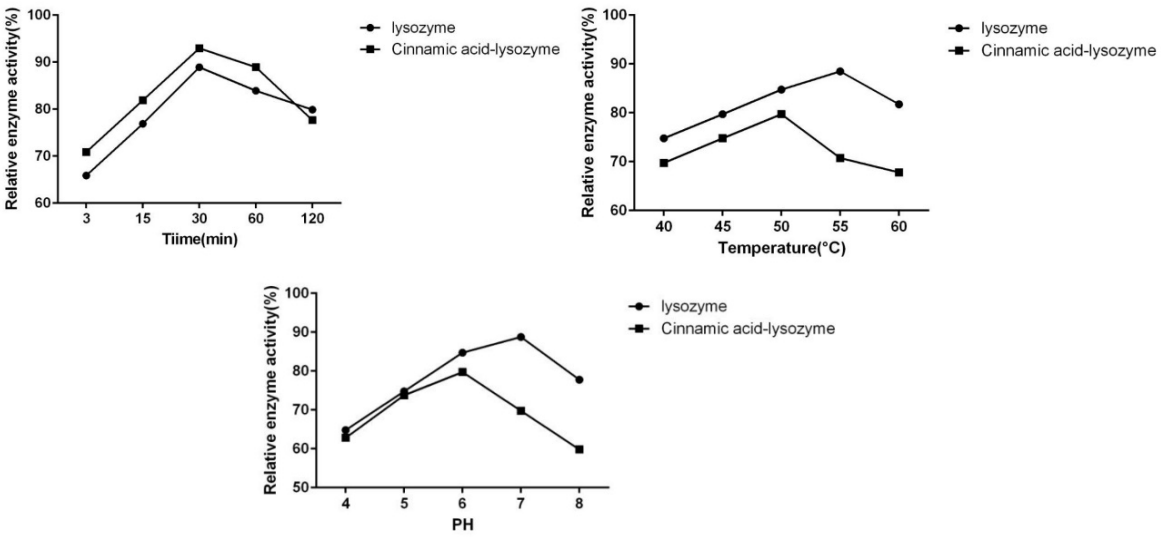


FIGURE 2. The Optimal temperature, PH and reaction time of lysozyme and modified lysozyme

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).

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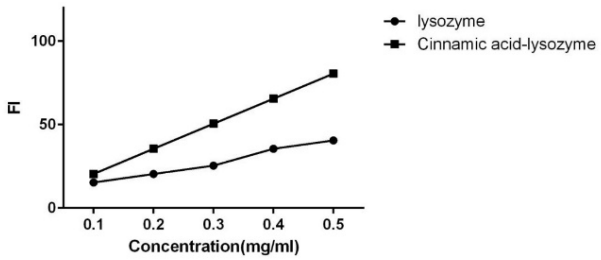

Sample	α-helix	β-sheet	β-turn	Random coil
lysozyme	28.79	22.38	20.18	28.65
Cinnamic acid-lysozyme	26.03	27.11	17.98	28.88

FIGURE 3. The hydrolysis of lysozyme and modified lysozyme and the comparison of secondary structure.

TABLE 2. Cytotoxicities 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	0.175±0.018	0.231±0.028	0.320±0.034	0.451±0.049

TABLE 3. 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	0.359±0.035
E.coli.	0.738±0.071	0.718±0.069	0.708±0.065	0.327±0.033	0.292±0.034

DISCUSSION

As shown in figure 1, bacteriostatic effects, and the minimum bacteriostasis concentration for cinnamic acid - modified lysozyme > lysozyme > Cinnamic 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[7-8]. PH = 6, the temperature is 50°C, the response time of 30 min strongest Cinnamic acid modified lysozyme activity (figure 2). 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[9]. Three kinds of enzyme surface hydrophobicity of the index of cinnamic acid modified lysozyme > lysozyme > cinnamic acid (figure 3).According to the structural characteristics and covalent binding characteristics of the three acids, the covalent binding of the natural enzymes and the three acids is the covalent binding of the amidogen of the enzyme molecules and the carboxyl of the organic acids[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). In addition, the excessively high concentration of pure

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lysozymes leads to a low content of the actually cross-linked lysozymes and decreases the encapsulation efficiency. 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) , according to their antibacterial activity against escherichia coli and staphylococcus aureus [11-12].

In conclusion, we have optimized the design of modified lysozymes with success and prepared the translucent balsa-modified lysozyme dressing characterized by strong antibacterial ability, stable and persistent release, non-cytotoxicity.

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