论文题目：蛋清中溶菌酶的提取、分离条件优化和性质实验

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摘 要

（注：我们希望锻炼英文，所以希望把正文写成英文版的，但因为毕设格式和英文文章的文内参考文献格式冲突，故按照英文的。希望老师给出建议！）

关键词：溶菌酶；提取；分离纯化；条件优化

Title: Optimization of Extraction, Separation Conditions of Lysozyme from Egg White and Characterization

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ABSTRACT

KEY WORDS: Lysozyme; Extraction; Separation and Purificaiton; Optimization

目 录

1 Preface 1
1.1 Physicochemical Properties 1
1.1.1 Structure and Mechanism 1
1.1.2 Function and Applications 2
1.2 Discussion on the Application 2
1.2.1 Applications in food industry 3
1.2.2 Clinical applications 3
1.3 Applications of Lyzoenzymes 4
1.3.1 Applications in Food and Fermentation Industry 4
1.3.2 Applications in Medical Industry 5
1.3.3 Other Applications 5
1.3.4 Limitations 6
2 Lysozyme Extraction, Isolation and Purification Experiments and Condition Optimization Methods 7
2.1 Experimental Setup 7
2.2 Experimental reagents and equipments 7
2.2.1 Pre-treatment of egg white samples 7
2.2.2 purification process 7
2.2.3 concentration measurement 7
2.2.4 Antibacterial test 8
2.3 Separation and Purification 8
3 溶菌酶的提取、分离纯化实验的结果分析 9
3.1 溶菌酶的提取条件 9
3.2 溶菌酶的分离纯化条件 9

4　结论与展望 10

致谢 13

1 Preface

1.1 Physicochemical Properties

Lysozyme (EC 3.2.1.17) is a protein existing in animals, plants, bacteria, and viruses. It can be found in neutrophils, macrophage granules, serum, saliva, milk, honey, and eggs. The enzyme hydrolyzed β-1,4 glycosidic bond between N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) of cytoderm peptidoglycan (PG) in Gram-positive and Gram-negative bacteria(Gajda Bugla-płoskońska, 2014). C-type lysozyme of egg white is a model for studying protein structure and function.

1.1.1 Structure and Mechanism

The three-dimensional structure of lysozyme was first resolved in 1965 by X-ray(Blake et al., 1965). Lysozyme consists of 129 amino acids cross-linked by 4 disulfide bonds, and lysozyme has two main domains. The α domain of the molecule is mainly composed of α helix, while the β domain contains β fold and helix. The active site is in the gap between the two domains.

There are two catalytic mechanisms to explain lysozyme. According to the Phillips mechanism, two residues Glu35 (glutamic acid) and Asp52 (aspartic acid) play an important role. The terminal proton of Glu35 is transferred to the O atom of the glycosidic bond between two adjacent sugar residues, which leads to cleavage of glycosidic bond and formation of a carbocation. The positive charge of the carbocation is stabilized by the negative charge of Asp52 until the hydroxide ion binds to the positive C atom and Glu35 is protonated. Another is Intermediate theory. Like all other retained β-glucosidases, egg white lysozyme proceeds through the formation of covalent intermediates and subsequent decomposition rather than through the formation of long-lived ion pairs(Vocadlo et al., 2001). The latest research supports the Phillips mechanism more(Held Van Smaalen, 2014).

Picture: (Ercan Demirci, 2016)

1.1.2 Function and Applications

Lysozyme has broad-spectrum resistance to both Gram-negative and Gram-positive bacteria. The bactericidal ability of lysozyme is not only due to its catalytic activity but also due to its cationic and hydrophobic characteristics (Pellegrini et al., 1992). Chemical modifications such as oleoyl chloride(Evran et al., 2010) and Na2SO3(Liu et al., 2018) can enhance the hydrophobic properties of lysozyme and enhance its antibacterial effect. Research(Ibrahim et al., 2001) proved that the helix-loop-helix domain located in the 87–114 sequence of lysozyme and its C-terminal helix domain passed through the outer membrane and damaged the inner membrane through self-promoting absorption, thus killing Gram-negative bacteria. In the human body, lysozyme degrades bacteria not only by directly killing bacteria but also by releasing immune regulatory bacterial ligands including PG fragments to participate in the regulation of the immune system(Ragland Criss, 2017). PG fragment can eliminate bacteria by enhancing the activation of phagocytes.

Egg white is composed of lysozyme, accounting for about 0.5% of its weight. At present, egg white lysozyme occupies a dominant position in the market, and its main limitations are high recovery cost, low activity, and immunological problems (Ercan Demirci, 2016). This immunological problem is dominated by IgE(Urisu et al., 2015), and people who are allergic to eggs will cause immunological problems, so the production of human lysozyme is also an important research direction. At present, the human lysozyme gene is expressed in heterologous organisms, such as rice(R.Wilken, 2011) and yeast(Huang Demirci, 2009). Studies in rice have proved that the manufacturing cost ($/g) is the same as that of eggs(R.Wilken, 2011).

1.2 Discussion on the Application

Given the ability to lyse the cell wall of bacterial, lysozyme has been attracting immense attention as a kind of environment-friendly or organs-friendly antimicrobial. As we have discussed before, it has nowadays more and more applications in the food industry and clinical procedure. In this part, we will discuss it’s applications within our preparations and capabilities.

1.2.1 Applications in food industry

Our first focus is on the food industry. Since the 1800s when Napoléon launched his strategy to conquer Europe, the storage or preservation of food has become a major question in the food industry. Sealing food in cans, High-temperature treatment, Pasteurization, numerous methods had risen. And in the last, people introduced chemical additives to the food industry. Efficient as it is, nowadays people are having less tolerance in that chemical industry. Due to the conception of eating healthy, they prefer so-called ”Non-additive food” to chemical treated food. But the lack of bacterial inhibition will easily make it a perfect bacterial petri dish. The chemical hazard vanishes, but the microbial hazard just arises. We need another method!

So we cast our sight to the biological method to preserve food. In our case, we are planning to introduce lysozyme to food packing and food additives. As for the food packing, we are going to distribute the lysozyme agent, in gluten(Conte et al., 2006) or onto a chitosan powder, on the food packages, mostly LDPE, these methods have been taken into practice(Borzooeian et al., 2017). And its function to extend to the shelf life of foods had been proved.(Lian et al., 2012; Alhazmi et al., 2014) our points of view, this kind of application suits our capability very well, and we have put it into our first consideration.

Another important application in the food industry is the food additive. We can add some lysozyme into specific easy-deteriorating foods，for example wurst,can-foods, and diary. The addition of lysozyme will significantly extend the preserve half-life of food, these lysozymes are presented into chitosan particals(Wu et al., 2017), this will not only protect the original flavour of the food but also enhance its ability to inhibit bacterial emerging.

1.2.2 Clinical applications

The lysozyme can also play an impressive role in the clinical procedure. Like the food industry, medical is also a battel against bacterial. In 1676, Anton van Leeuwenhoek observed bacteria and other microorganisms, using a single-lens microscope of his design. In 1796, Edward Jenner developed a method using cowpox to successfully immunize a child against smallpox. The same principles are used for developing vaccines today. Following on from this, in 1857 Louis Pasteur also designed vaccines against several diseases such as anthrax, fowl cholera and rabies as well as pasteurization for food preservation. In 1867 Joseph Lister is considered to be the father of antiseptic surgery. By sterilizing the instruments with diluted carbolic acid and using it to clean wounds, post-operative infections were reduced, making surgery safer for patients. In 1929 Alexander Fleming developed the most commonly used antibiotic substance both at the time and now: penicillin.(Brock et al., 2003).The emerge of antibiotic medicine start a new era for human, we can sometimes beat the infection of microbes.

But there still exists a fatal problem: ALLERGY. Some antibiotics will lead to an acute allergic phenomenon, which is sometimes fatal. So we come up with this idea to introduce lysozyme to health-care products. We want to introduce it in for instance dentifrices, mouth-rinses, moisturizing gels, chewing gums or such sterilization products.(Tenovuo, 2002) We try to develop a kind of lysozyme covered bandage in which the lysozyme exist in gel, or in other advanced status, such as carbon nanotubes. These are our prospects of the clinical application of lysozyme.

1.3 Applications of Lyzoenzymes

Today, lysozymes has been widely applied to food, medical and biological industry. Here we conducted a brief summary of current reasearch on application of lyzozymes. We find that our two ideas are practical and potentially valuable in people's daily life.

1.3.1 Applications in Food and Fermentation Industry

Natural lyzozymes can repress bacterial growth without undermining our health, thus they are mainly used as preservatives. Zhai et al. (2015) demonstrate in their review that, lyzozymes can prevent cheese from microorganism-caused swelling during the production; they also have an outstanding effect in retaining the freshness of meat product conbined with other natural preservatives. Quan (2006) and Zhai et al. (2015) all show that lyzozymes are added into Japanese sake (a kind of low wine) to replace salicylic acid or SO_2 in as the preservative. Microorganisms are initiators of aquatic products' rotting. Ren et al. (2013) point out in their review that lyzozymes may do better than instant cool storage with the help of some other techniques (like ultrahigh pressure).

1.3.2 Applications in Medical Industry

Lyzozymes are a conponent of the second line of immune defense in our body for their nonspecific bactericidal effect. Movever, people are adding extra lyzozymes to strengthen our immune system or cure inflammation and infection. Lyzozymes can also improve theraputic effect of various kinds of drugs.

According to (Quan, 2006) and (Zhai et al., 2015), lyzozymes can regulate gut microbes by specifically killing putrefactive ones and selectively increase the number of bifidobacterium, which is a significant intestinal probiotic. Therefore, lyzozymes bring a remission to enteritis and enhance the immune system. This is pretty helpful for susceptible infants. Adding lyzozyme to milk is widely applied as a quality-improvement strategy. Lyzozymes extracted from egg white are also made into industralized mouth wash, which can inhibit over 99% *Escherichia coli* and *Staphylococcus aureus* (Unk, 2020).

He et al. (2008) also reviewed applications in medical industry, listed as follows:

- Inhibition of dental caries growth;
- Treatment of surface ulcers, fungal infection, burn wound infection and herpes (with a better than traditional antifungal drugs);
- Treatment of SARS virus infection;
- Potential as a tumor treatment adjuvant.

1.3.3 Other Applications

Attaching lyzozymes to polymers is of great application value and worth deeper study. Wang and Kuang (2020) reviewed applications of those composites. Polymers like polysaccharide, protein and polyphenol etc. enhance lyzozymes' bactericidal activity, stability and range of application.

With their excellent biocompatibility, Lin (2015) constructed a lyzozyme/pectin complex for drug delivery with satisfying results.

Lyzozymes play an important role in gene engineering as a kind of tool enzyme. They remove the cell wall of Gram-positive bacteria and protoplast is obtained to be used for cell fusion or cellular matter extraction (Quan, 2006).

1.3.4 Limitations

Although lyzozymes are extensively studied, there remains problems to be solved. As a natural product, the separation and purification method should be optimized. Industrialized production is still under developing and the cost is stiil high Zhai et al. (2015). Zhao et al. (2009) state in their review that natural lyzozymes don't have a wide antibacterial spectrum, which cannot cover most Gram-negative bacteria especially. Some propose that protein-modification might be useful, but the safety cannot be guaranteed and protein structure should be studied. At the same time, cooperation, optimal adding proportion and working condition between lyzozymes and other preservatives are still under further research.

2 Lysozyme Extraction, Isolation and Purification Experiments and Condition Optimization Methods

2.1 Experimental Setup

2.2 Experimental reagents and equipments

Fresh eggs, wall-warming micrococcus

NaCl, NaH2PO4, NaOH, (NH4)2SO4, concentrated hydrochloric acid

Comas Brilliant Blue G250, Comas Brilliant Blue R250, 0.45 μm filter membrane, CM Sepharose Fast Flow (GE), Sephacryl-S200 (GE), constant flow pump, UV monitor, UV-Vis spectrophotometer, fraction collector, chromatography data acquisition and processing system, glass chromatography column, centrifuge, acidometer.

2.2.1 Pre-treatment of egg white samples

Wash the egg shell, dry the outside of the shell, gently crack the shell, pour the egg whites into a beaker, with the yolk intact, strain the egg whites through 2 layers of gauze to remove the umbilical chunks and shell fragments, then collect the egg white. Then dilute the egg whites with Buffer1 to 1.5 times the original volume and stir with a glass rod, filter with 6 layers of gauze, adjust the pH of the filtrate with dilute hydrochloric acid to 4.5, rest for 20 min, centrifuge at 12 000 rpm for 10 min.

2.2.2 purification process

Purification of lysozyme by cation exchange

SDS-PAGE to check the purification effect

Molecular Sieve Chromatography

2.2.3 concentration measurement

The concentrations of proteins in each sample were determined using the Comas Brilliant Blue G250 staining method.

2.2.4 Antibacterial test

Test bacteria (Bacillus coccoidus, Bacillus cereus, E. coli), delineate on solid LB plate, cultivate at 35℃ for about 16h, pick single colony and inoculate on liquid LB medium, cultivate at 35℃ for about 12h at 200rpm, get the liquid after activation, add the same concentration of sample and measure the diameter of inhibition loop.

2.3 Separation and Purification

3　溶菌酶的提取、分离纯化实验的结果分析

3.1　溶菌酶的提取条件

3.2　溶菌酶的分离纯化条件

4 结论与展望

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致　　谢

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