

论文题目：蛋清中溶菌酶的提取、分离条件优化和性质实验

学生姓名：高旭帆，郭骐瑞，程肖然，黎博琛

指导教师：孔宇

摘 要

溶菌酶是一种能水解 N-乙酰胞壁酸 (NAM) 和 N-乙酰氨基葡萄糖之间的 β -1,4 糖苷键，从而破坏细菌细胞壁的酶，目前已广泛应用于食品、医疗、生物等产业。但是这种天然产物的分离纯化方法还有待完善。本研究从蛋清中提取溶菌酶，用阳离子交换和 SDS-PAGE 的方法进行纯化，用考马斯亮蓝 G250 测定溶菌酶浓度，并检测其抗菌活性。我们希望这些溶菌酶能够应用于食品储存和医疗保健产品。

（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

Name: Xufan Gao, Qirui Guo, Xiaoran Cheng, Bochen Li

Supervisor: Yu Kong

ABSTRACT

Lysozymes are a kind of enzyme which can hydrolyze β -1,4 glycosidic bond between N-acetyl muramic acid (NAM) and N-acetyl glucosamine, thus destroy bacterial cell wall. Nowadays, they have been widely applied to food, medical, and biological industries. However, separation and purification methods still needs perfection for this natural product. In this work, we extracted lysozymes from egg white, purified them with cation exchange and SDS-PAGE, determined their concentration using Comas Brilliant Blue G250, and tested their antibacterial activity. We hope these lysozymes could be applied to food storage and health-care products.

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

Contents

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	3
1.2.1	Applications in Food Industry	3
1.2.2	Clinical Applications	4
1.3	Applications of Lysoenzymes	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
2.1.1	Experimental Reagents and Equipments	7
2.1.2	Pre-treatment of Egg White Samples	7
2.1.3	Purification Process	7
2.1.4	Concentration Measurement	8
2.1.5	Antibacterial Test	8
3	Analysis of Results of lysozyme Extraction, Separation and Purification Experiments	9
3.1	Extraction Conditions	9
3.2	Separation and Purification Conditions	9

4 Conclusion.....	10
致谢	15

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 and 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 and Van Smaalen, 2014).

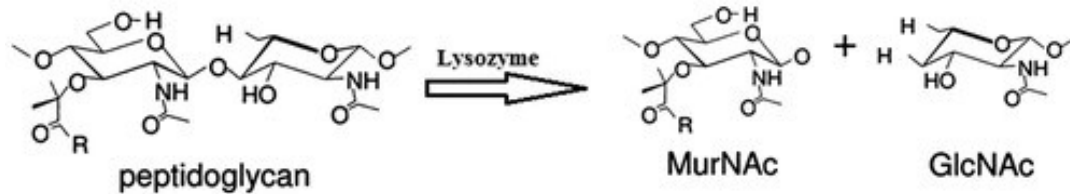


Figure 1-1 The Reaction. Extracted from Ercan and Demirci (2016)

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 and 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 and 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 and Nikolov, 2011) and yeast (Huang and Demirci, 2009). Studies in rice have proved that the manufacturing cost (\$/g) is the same as that of eggs (R.Wilken and Nikolov, 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 procedures. In this part, we will discuss its 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 (Alhazmi et al., 2014; Lian et al., 2012). 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, such as wurst, can-foods, and dairy. 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 battle against bacteria. 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 emergence of antibiotic medicine starts 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 exists 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 Lysozymes

Here we conducted a summary of current research on the application of lysozymes. We find that our two ideas are practical and potentially valuable in our daily life. Today lysozymes have been widely applied to food, medical, and biological industries.

1.3.1 Applications in Food and Fermentation Industry

Natural lysozymes can repress bacterial growth without undermining our health thus they are mainly used as preservatives. Zhai et al. (2015) demonstrate in their review that lysozymes

can prevent cheese from microorganism-caused swelling during the production; they also have an outstanding effect in retaining the freshness of meat product combined with other natural preservatives. Quan (2006) and Zhai et al. (2015) all show that lysozymes are added into Japanese sake (a kind of low wine) to replace salicylic acid or SO₂ in as the preservative. Microorganisms are initiators of aquatic products' rotting. Ren et al. (2013) point out in their review that lysozymes may do better than instant cool storage with the help of some other techniques like ultrahigh pressure.

1.3.2 Applications in Medical Industry

Lysozymes are a component of the second line of immune defense in our body for their nonspecific bactericidal effect. Moreover, people are adding extra lysozymes to strengthen our immune system or cure inflammation and infection. Lysozymes can also improve the therapeutic effect of various kinds of drugs.

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

He et al. (2008) also reviewed applications in the 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 lysozymes to polymers is of great application value and worth deeper study. Wang and Kuang (2020) reviewed applications of those composites. Polymers like polysac-

charide, protein, and polyphenol, etc. enhance the bactericidal activity, stability, and range of application of lysozymes. With their excellent biocompatibility, Lin (2015) constructed a lysozyme/pectin complex for drug delivery with satisfying results.

Lysozymes also 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 for cell fusion or cellular matter extraction (Quan, 2006).

1.3.4 Limitations

Although lysozymes are extensively studied, there remain problems to be solved. As a natural product, separation and purification methods still needs perfection. Industrialized production is still under development and the cost is still high Zhai et al. (2015). Zhao et al. (2009) state in their review that natural lysozymes don't have a wide antibacterial spectrum. Some propose that protein-modification might be useful, but the biosafety cannot be guaranteed and protein structure should be studied. At the same time, cooperation, optimal adding proportion, and working condition between lysozymes and other preservatives are still under further research (Zhai et al., 2015).

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

2.1 Experimental Setup

The following procedures are based on Li-li et al. (2017); Quan (2006); Yijun et al. (2020).

2.1.1 Experimental Reagents and Equipments

Material: Fresh eggs, wall-warming micrococcus

Reagents: 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.

In addition, we used different ratios of NaCl to phosphate to configure Buffer for different pH values.

2.1.2 Pre-treatment of Egg White Samples

We start with an intact egg, wash the shell, and dry the outside of the shell. Afterwards, we gently crack the shell and pour the egg whites into a small beaker. With the yolk intact, we strain the egg whites through 2 layers of gauze to remove any umbilical chunks and broken shells, collecting the liquid. The egg whites are then diluted to 1.5 times their original volume with Buffer1 and stirred with a glass rod before being filtered through 6 layers of gauze. Finally, the supernatant was filtered through a 0.45 µm membrane and the filtrate was obtained.

2.1.3 Purification Process

We attempt to purify the lysozyme using cation exchange and then tested the purification by SDS-PAGE. After finding a suitable elution peak, we can use salt chromatography with re-suspension to precipitate our desired enzyme from the sample. After purification by molecular

sieve chromatography, we again check the results of the sieve purification by SDS-PAGE.

2.1.4 Concentration Measurement

We choose to use the Comas Brilliant Blue G250 staining method to determine the protein concentration of each sample.

2.1.5 Antibacterial Test

We first select the test species (*Bacillus coccoidus*, *Bacillus cereus*, *E. coli*), scribed on solid LB plate, incubated at 35 °C for 16h or so. After that, single colonies can be inoculated into liquid LB medium. After incubation under suitable conditions, we obtain a series of post-activation fluids. Finally, we directly add the same concentration of the sample obtained in previous experiments. The inhibition activity of lysozyme is determined by measuring the diameter of its inhibition loop.

3 Analysis of Results of lysozyme Extraction, Separation and Purification Experiments

3.1 Extraction Conditions

3.2 Separation and Purification Conditions

4 Conclusion

References

- New Functional Egg Products: Egg White Lysozyme Mouthwash. *China Rural Science & Technology*, 04:48, 2020.
- Alaa Alhazmi, Johnathan Warren Stevenson, Samuel Amartey, and Wensheng Qin. Discovery, Modification and Production of T4 Lysozyme for Industrial and Medical Uses. *Int. J. Biol.*, 6(4), 2014. ISSN 1916-9671. doi: 10.5539/ijb.v6n4p45.
- C Blake, D Koenig, G Mair, A North, D Phillips, and V Sarma. The three-dimensional structure of hen eggwhite lysozyme. *Nature*, 206(49):757–761, 1965.
- Z. Borzooeian, M. E. Taslim, G. Borzooeian, O. Ghasemi, and M. Aminlari. Activity and stability analysis of covalent conjugated lysozyme-single walled carbon nanotubes: Potential biomedical and industrial applications. *RSC Adv.*, 7(77):48692–48701, 2017. ISSN 20462069. doi: 10.1039/c7ra07189b.
- Thomas Dale (viaf)108182990 Brock, Michael T (viaf)27221308 Madigan, John M (viaf)24825109 Martinko, and Jack (viaf)115234898 Parker. *Brock biology of microorganisms*. Upper Saddle River (N.J.) : Prentice-Hall, 10th ed. edition, 2003. ISBN 0130491470. URL <http://lib.ugent.be/catalog/rug01:000745286>.
- A. Conte, G. G. Buonocore, A. Bevilacqua, M. Sinigaglia, and M. A. Del Nobile. Immobilization of lysozyme on polyvinylalcohol films for active packaging applications. *J. Food Prot.*, 69(4):866–870, 2006. ISSN 0362028X. doi: 10.4315/0362-028X-69.4.866.
- Duygu Ercan and Ali Demirci. Recent advances for the production and recovery methods of lysozyme. *Critical Reviews in Biotechnology*, 36(6):1078–1088, 2016. ISSN 15497801. doi: 10.3109/07388551.2015.1084263.
- Serap Evran, Ihsan Yaşa, and Azmi Telefoncu. Modification of lysozyme with oleoyl chloride

- for broadening the antimicrobial specificity. *Preparative Biochemistry and Biotechnology*, 40(4):316–325, 2010. ISSN 10826068. doi: 10.1080/10826068.2010.488528.
- Ewa Gajda and Gabriela Bugla-płoskońska. Lizozym – występowanie w przyrodzie , właściwości biologiczne i możliwości zastosowań Lysozyme – occurrence in nature , biological properties and possible applications. *Postepy Hig Med Dosw (Online)*, 21(68): 1501–1515, 2014.
- Jin-hua He, Yu Liu, Guan-jie Liu, Lin-na Li, Hai-cheng Li, and Li-juan Wang. The Applications and Advances of Lysozyme in Medicine. *Today's Pharmacology*, 02:16–19, 2008.
- Jeanette Held and Sander Van Smaalen. The active site of hen egg-white lysozyme: Flexibility and chemical bonding. *Acta Crystallographica Section D: Biological Crystallography*, 70(4):1136–1146, 2014. ISSN 09074449. doi: 10.1107/S1399004714001928.
- Eric Lu Huang and Ali Demirci. Enhanced Human Lysozyme Production by *Kluyveromyces lactis*. *Food Bioprocess Technol*, 2(2):222–228, 2009. doi: 10.1007/s11947-008-0062-1.
- Hisham R Ibrahim, Ursula Thomas, and Antonio Pellegrini. A Helix-Loop-Helix Peptide at the Upper Lip of the Active Site Cleft of Lysozyme Confers Potent Antimicrobial Activity with Membrane Permeabilization Action. *Journal of Biological Chemistry*, 276(47):43767–43774, 2001. ISSN 00219258. doi: 10.1074/jbc.M106317200.
- Zhang Li-li, Li Chen-xi, and Chang Yun-kang. 张黎丽, 马梦琳, 李晨熙, 常允康 * (. 3 (1):3–6, 2017.
- Zi Xuan Lian, Zhong Su Ma, Jing Wei, and Huan Liu. Preparation and characterization of immobilized lysozyme and evaluation of its application in edible coatings. *Process Biochem.*, 47(2):201–208, 2012. ISSN 13595113. doi: 10.1016/j.procbio.2011.10.031. URL <http://dx.doi.org/10.1016/j.procbio.2011.10.031>.
- Liu-feng Lin. *Construction of Lysozyme/Pectin Complex and Its Application in Delivery Systems for Nutrition and Drug*. Master, Huazhong Agricultural University, 2015.

REFERENCES

- Jihong Liu, Ning Wang, Yaping Liu, Yongguo Jin, and Meihu Ma. The antimicrobial spectrum of lysozyme broadened by reductive modification, 2018. ISSN 15253171.
- A. Pellegrini, U. Thomas, R. von Fellenberg, and P. Wild. Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. *Journal of Applied Bacteriology*, 72(3):180–187, 1992. ISSN 13652672. doi: 10.1111/j.1365-2672.1992.tb01821.x.
- Yu-tong Quan. *Studied on the Extraction and Activities of Lysozyme from Egg White*. Doctor, Jilin University, 2006.
- Stephanie A Ragland and Alison K Criss. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS pathogens*, 13(9):e1006512, 2017.
- Xi-ying Ren, Ya-qin Hu, Qing-lan Hu, Yang Shui-bing, Hai-xia Yu, Gang Zheng, and Yang Zhi-jian. Application of lysozyme in preservation of aquatic products. *Science and Technology of Food Industry*, 34(08):390–395, 2013. doi: 10.13386/j.issn1002-0306.2013.08.084.
- Lisa R. Wilken and Zivko L. Nikolov. Process evaluations and economic analyses of recombinant human lysozyme and hen egg-white lysozyme purifications.pdf. *Biotechnol Prog*, 27(3):733–734, 2011.
- J. Tenovuo. Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: Efficacy and safety. *Oral Dis.*, 8(1):23–29, 2002. ISSN 1354523X. doi: 10.1034/j.1601-0825.2002.1o781.x.
- Atsuo Urisu, Y Kondo, and I Tsuge. Hen 's Egg Allergy. *Chem Immunol Allergy*, 101:124–130, 2015. doi: 10.1159/000375416.
- David J Voadlo, Gideon J Davies, Roger Laine, and Stephen G Withers. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. *Nature*, 412(6849):835–838, 2001. ISSN 00280836. doi: 10.1038/35090602.
- Le-chuan Wang and Hai-rui Kuang. Research Progress of High Molecular Polymer in Lysozyme Immobilization. *Food Research And Development*, 38(10):87–93, 2020.

- Tiantian Wu, Chunhua Wu, Shalu Fu, Liping Wang, Chunhong Yuan, Shiguo Chen, and Yaqin Hu. Integration of lysozyme into chitosan nanoparticles for improving antibacterial activity. *Carbohydr. Polym.*, 155:192–200, 2017. ISSN 01448617. doi: 10.1016/j.carbpol.2016.08.076. URL <http://dx.doi.org/10.1016/j.carbpol.2016.08.076>.
- L I U Yijun, Zhao Yuxuan, G A O Songmin, Wang Ya, and H A O Shuailin. Improvement of lysozyme separation and purification of egg white. 2020. doi: 10.16791/j.cnki.sjg.2020.08.043.
- Wen-yi Zhai, Gui-xiang Zhang, Bing-wen Zhang, and Qiang Wu. Scientific Interpretation of Chinese Traditional Food: Steamed Buns. *Food and Nutrition in China*, 21(12):28–31, 2015.
- Dian-bo Zhao, Yan-hong Bai, Zhang Xiao-yan, and You-jun Wu. Research Progress of Modification of Natural Biological Preservative Lysozyme. *China Food Additives*, (05):200–204, 2009.

致 谢

谨此向孔宇教授致以衷心的感谢和崇高的敬意！