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

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

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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 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).

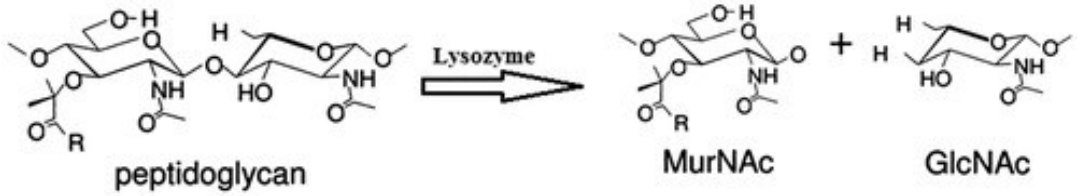

Figure 1-1 The Reaction. Extracted from Ercan and Demirci (2016)

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 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 (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 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

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 SO_2 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. Lyzozymes extracted from egg white are also made into industralized 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 Principles

2.1.1 Ion exchange chromatography

Ion exchange chromatography is a widely applied experimental technique to separate substances. We firstly set up a glass column and add the original solution with a solute of interest into it. Fixing a kind of high molecular weight (HMW) substances called ion exchanger on the inner wall of the glass column, their exchangeable groups (or ions) go into the solution, and some components of the solutes are absorbed onto the ion exchanger. (Here we apply cation exchange chromatography, where the exchangeable group is cation) This is call exchange re action. The reaction is reversible and follows Le Chatelier’ s principle. When an exchangeable group disassociates from the column wall and its concentration in the solution rises, the solution flows away and a new solution without this group or ion comes, pushing the reaction going towards the positive direction. Meanwhile, the solute we are interested in is all absorbed onto the column wall. Then we use another kind of solution to wash off (elute) this solute and get a pure solution of it.

The experiment can be divided into four phases:

1) balance: adding a solution to achieve the balance between ion exchanger and its exchangeable groups;

2) absorption: the exchange reaction;

3) elution: using buffers with different concentration to wash off substances in the order of low to high affinity;

4) regeneration: use the original buffer (the solution to balance columns) to recover the columns.

The effectiveness of separation is determined by the affinity of exchangeable groups and

the solute. Furthermore, this affinity may change as the physicochemical properties (like pH, salt concentration) change. So a careful choice of separation condition is important. Here we add NaCl into the buffer to lower the solubility and promote the absorption. Thus, other components will be absorbed in a farther position, making it easy to separate them.

2.1.2 SDS-PAGE

SDSPAGE(sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a commonly used method to separate proteins with different molecular weights. Proteins are mixed with SDS which denaturate them into rodlike macromolecules. SDS is a highly negativecharged micromolecule. It binds to proteins according to molecular weight (MW) of the protein and forms a negativecharged complex. We put protein into an electrostatic field, and their only difference is their size (MW).

Proteins will firstly be concentrated and pass through holes on PAGE, which is a crosslinking polymer. The bigger a protein is, the harder it moves forward and passes the structure, and the later it reaches the anode. Thus we can separate different sized proteins. Using the Comas Brilliant Blue R250 staining method, we can see the distribution of proteins. If the band of our target protein is narrow and light without other bands near it, our product is pure, and its concentration is high.

2.1.3 Salting Out

This method is often used to separate and purify biomarcomolecules. Adding high concentration inorganic salt (like $(NH_4)_2SO_4$, NaCl) into the solution of HMW substances will decrease their solubility and precipitate them. Those salts dissolve very well, deconstructing the water layer on the surface of HMW substances and neutralizing their charge. Thus, they are separated from other solutes.

2.1.4 Gel Filtration Chromatography

This is another usual separation method, which is extensively used because of its mild operational conditions. It also uses a reticular polymer (usually glucan or agarose), but the principle is different from SDS-PAGE. The bigger a protein is, the harder it is trapped in the hole, and the faster it reaches theother end of the column. Other micromolecules or smaller

proteins stay in the hole and are separated from target proteins.

The experiment can be divided into four steps:

1) swelling: put the dry gel into water or eluent;

2) filling: add gel to the column evenly (very important);

3) balancing, and loading sample;

4) finding the smaple peak, and collecting;

5) recycling the gel.

The effectiveness of separation is mainly decided by molecular weight and affected by other conditions. We also need to carefully choose the type of gel, the length of the column, the buffer and so on.

2.2 Experimental Supplies

See Table 2-1,2-2,2-3,and 2-4.

Table 2-1 glass instrument

Name	Specifications	Quantity
Beaker	50	2
	200	2
	500	2
Glass rod		2
Funnel		1
Quartz cuvette		4
Gel column		1
26 mm glass chromatography column		1
Test tube		6
Iron frame platform		1
Pipette	1000	1
	200	1
	20	1

2.3 Experimental Setup

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

Table 2-2 instruments

Name	Quantity
Ultraviolet visible spectrophotometer	1
Protein purification system Including pump, collector and detector	1
EPS 601 DC regulated power supply	1
Vertical plate electrophoresis tank	1
Freeze vacuum dryer	1
Ph meter	1

Table 2-3 consumables

Name	Specifications	Quantity
hen eggs		40
Gauze	package	1
0.45 μm filter membrane	package	1
1.5 mL centrifuge tube	package	1

Table 2-4 reagents

Name	Specifications	Quantity
50% CM Sepharose Fast Flow (ion exchange suspension)	ml	50
$(NH_4)_2SO_4$	g	100
50% Sephacryl-S200 medium		
SDS	g	50
Ammonium persulfate (AP)	g	5
TEMED	ml	2
Tris	g	20
Glycine	g	94
HCl	ml	200
n-butanol	ml	5
0.5% bromophenol blue	ml	10
50% glycerol	ml	100
Coomassie Brilliant Blue R250	ml	20
5%β- mercaptoethanol	ml	10
Methanol	L	1
Glacial acetic acid	ml	200
NaOH	g	20
0.5mg/ml of standard protein solution (bovine serum albumin solution)	ml	5
Coomassie Brilliant Blue G250	ml	100
NaCl	g	100
NaH_2PO_4	g	500
Na_2HPO_4	g	200
Micrococcus wallichii powder	g	5

2.3.1 Experimental Reagents and Equipments

Material: Fresh eggs, wall-warming micrococcus

Salt solutions: NaCl, NaH_2PO_4, NaOH, $(NH_4)_2SO_4$, concentrated hydrochloric acid

Reagents: 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.

2.3.2 Buffer

- Buffer1：pH=4.5，0.1 mol/L phosphate，50 mmol/L NaCl
- Buffer2：pH=4.5，0.1 mol/L phosphate，200 mmol/L NaCl
- Buffer3：pH=4.5，0.1 mol/L phosphate，500 mmol/L NaCl
- Buffer4：pH=4.5，0.1 mol/L phosphate，1 mol/L NaCl
- Buffer5：pH=7.0，0.1 mol/L phosphate，50 mmol/L NaCl

2.3.3 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.

2.3.4 Purification Process

We attempted 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 resuspension 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.

1) Ion exchange chromatography

We took about 15 mL of 50% CM Sepharose Fast Flow ion exchange suspension and loaded it onto a 26 mm diameter glass column to check the seal.

After that, we used Buffer1 to balance the ion exchange column, adjusted the constant flow rate of the constant flow pump to 1 mL/min, balanced for about 30 min (about 4-5 column volumes), and finally stabilized the UV absorption curve at the baseline.

We then loaded Sample1 at a flow rate of 0.5 mL/min and rinsed with Buffer1 at a flow rate of 1 mL/min. Record the OD280-time curve and observe the elution peaks as they occur, stopping the flush when OD280 drops close to baseline and remains unchanged.

We then elute with Buffer2 at a flow rate of 2 mL/min and open the fraction collector for fraction collection (1 mL/tube).

When the OD280 returns to baseline, we switch to Buffer3 for elution at 2 mL/min flow rate.

After elution, we rinse 5 column volumes of regeneration medium with Buffer4 at a flow rate of 2 mL/min and then use Buffer1 at a flow rate of 1 mL/min to wash away the residual high salt solution in the column to complete column regeneration.

Finally, the elution peaks at different Buffer were obtained.

2) Using SDS-PAGE to detect purification effects

Denaturing polyacrylamide gels (15% separated gel, 4% concentrated gel) were prepared. We then electrophoresed a small amount of each fraction obtained from ion exchange chromatography separately and displayed the electrophoresis results using the Comas Brilliant Blue R250 staining method. We determined the lysozyme bands based on molecular weight and the purity of lysozyme in the eluted fraction based on the electrophoresis results, and selected the best elution peak as sample2.

3) Salting and resuspension

We dissolved 3.5 mg of (NH4)2SO4 powder in 10 mL of Sample2 in several small amounts and stirred with a glass rod while adding the powder to prevent local salt concentration from

excessive hetero protein salting. The suspension was divided into 1.5 mL centrifuge tubes and centrifuged at 12,000 rpm for 10 min, the supernatant from each tube was discarded. Finally, resuspend with 1 mL of Buffer5 and combine all the precipitates.

4) Molecular Sieve Chromatography

We took an appropriate amount of 50% Sephacryl-S200 medium and added Buffer5 at a flow rate of 0.8 mL/min to equilibrate the medium on a glass chromatography column. The sample was then loaded and eluted with Buffer5, while the fraction collector was opened for peak collection (1 mL/tube). Record OD280 - time curve. When the OD280 returns to base-line and elutes beyond one column volume, stop the elution. Finally determine the fraction absorption peak corresponding to the lysozyme.

5) Detection of molecular sieve effects by SDS-PAGE

We first observed the results of molecular sieve purification by SDS-polyacrylamide gel electrophoresis analysis, after which we selected a single fraction of the electrophoretic band and mixed it well as Sample3 (measured volume).

2.3.5 Concentration Measurement

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

2.3.6 Antibacterial Test

1) Preparation of standard solution for wall-dissolving micrococci

Dissolve the purchased micrococcal powder with Buffer5, grind it thoroughly in a steril-ized mortar, decant and dilute the substrate suspension to the desired absorbance value.

2) Determination of lysozyme activity

Add 2.5 mL of substrate suspension and 0.5 mL of standard enzyme solution to a 1 cm cell at 25℃, mix well and count the blanks, measure the absorbance every 15 s for a total of 6 times. pH=6.4).

Sample	Sample1	Sample2	Sample3
Micrococcus lysozyme	800	800	800
Buffer5	195	195	185
Sample volume	5	5	5

3) Definition of enzyme activity unit.

At room temperature 25°C and pH 6.2, at a wavelength of 450 nm, a decrease of 0.001 in absorption caused by each min is one enzyme activity unit, then the activity unit U per mg of lysozyme is

$$EnzymeActivity(\mathrm{U}\cdot\mathrm{mL}^{-1}) = \frac{OD_{420}(60) - OD_{420}(0)}{v_0} \times 10^6$$

2.4 Our Innovation

The extraction and the purification of lysozyme have been widely and deeply researched, there have existed many useful protocols and measurements concerning lysozyme. So to demonstrate our innovation, we cast our sight to the application of lysozyme.

As for the food industry, we are planning a Lysozyme-contained polygenic Sphere. This kind of product will present very attracting characteristics such as low health hazard, controlled lysozyme release and the most important: high stability. All these properties will make our product help the food industry improve their food’s half-shelf life without introducing hazardous chemicals. We have designed a polygon-sphere with unregular-shaped holes on it. The polygon-made skeleton will provide a stable foundation to the product while the unregular hole will provide adequate specific surface area to the efficient working of lysozyme.

In the articles we have referred to, they always introduce a soft or gel-like material due to the excellent controlled-release properties. We decide to use the hard polygenic material while equipping holes on it, we think that this will equip our product higher stability and get rid of the problems by the dissolve of the gel material.

Figure 2-1 a skecth of our polygon-sphere

Apart from the food industry, we also come up with ideas applying our products to medicine. We can inlay our microsphere to the bandage, and make our bandage present anti-microbe properties, which will minimize the frequency of re-applying the bandage.

The applications of lysozyme are being more and more popular recently, and we hope our methods will provide a new idea of applying lysozyme in our daily lives.

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

4 Conclusion

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