MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Studies on the maturation of glutamine transaminase modified by protease**

First (Bachelor's) level of higher education

Specialty 162 "Biotechnology and Bioengineering"

Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Kyiv 2024

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Educational and professional program Biotechnology

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«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Peng Hao

1. Thesis topic **Studies on the maturation of glutamine transaminase modified by protease**
Scientific supervisor Olena Okhmat, Ph.D., Assoc. Prof.

approved by the order of KNUTD "____"_____2024, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

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SUMMARY

Hao Peng. Studies on the maturation of glutamine transaminase modified by protease – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

Glutamine transaminase (glutamine transaminase) is a kind of enzyme that exists widely in many organisms. Its main function is to catalyze covalent cross-linking of proteins within or between molecules. And has development prospects in many fields. In order to promote the modification and maturation of glutamine transaminase, we selected a neutral protease from a variety of proteases at the initial stage of the experiment. This protease has stronger activity and better promotion effect on glutamine transaminase compared with other proteases. Therefore, we immobilized the neutral protease to realize the efficient maturation of glutamine transaminase.[2] Later, we calculated the specific improvement of glutamine transaminase by neutral protease, and set the control experiment variable as whether neutral protease was added or not. The results showed that the enzyme activity of the group with neutral protease was about 75% higher than that of the group without neutral protease. Neutral proteases exhibit high activity in neutral to weakly alkaline environments, with an optimal pH operating range of 6.5 to 8.0. The optimum temperature is 50℃ to 60℃. The incubation pH needs to reach pH8 to reach the optimal pH, and the incubation temperature needs to be maintained at about 30℃. Then, the immobilized neutral protease was compared with the free neutral protease, and it was found that the immobilized protease was significantly better than the unfixed group when incubated at pH 5 for 60 minutes. Immobilization improves thermal stability and pH stability. Immobilization significantly improved the thermal stability and pH stability of neutral protease. Finally, the reusability analysis of neutral protease was carried out, and the results showed that the immobilized enzyme had adaptability.[3]

Key words: Glutamine transaminase protease, enzyme activity, efficient expression.

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INTRODUCTION

In their quest to improve Transglutaminase (TGase) activity, the researchers conducted screening experiments on a variety of different proteases to identify which would most effectively promote TGase modification and maturation. In the experiment, TGase was co-cultured with different proteases and performed under consistent conditions to evaluate the specific effects of proteases on TGase activity. By measuring changes in TGase activity, the researchers found that neutral proteases were more effective in promoting TGase modification maturation than other proteases.

In addition, the immobilization of TGase is also a strategy to improve its performance in industrial applications. Immobilized TGase shows advantages in catalytic efficiency and reusability, which is important for reducing production costs and improving operation convenience.

In summary, the study of protease on TGase not only helps to understand the interaction between enzymes, but also provides new strategies and methods for the application of TGase in food processing, biomedicine and other fields. Through these studies, we can better utilize the catalytic properties of TGase and develop more high-quality products."

The relevance of the topic is The activity and stability of TGase are key factors in determining its effectiveness in industrial applications.

The purpose of the study is The proteases which can effectively enhance TGase activity were screened. The specific effects of protease treatment on TGase activity and stability were determined.The immobilization conditions of TGase were optimized to improve its performance in repeated use.

The objectives of the study of the research in this field aims to explore the potential effects of different types of proteases on TGase activity, as well as the molecular mechanisms of these effects. In addition, the study aims to determine

which conditions maximize the activity and stability of TGase, thereby providing guidance for the application of TGase in various industrial processes.

The object of the study – Transglutaminase (TGase).

The subject of the study TGase.

Research methods Enzyme immobilization technology.

The scientific novelty Enzyme immobilization technology.

The practical significance of the results By identifying proteases that can increase the activity of TGase, the catalytic efficiency of TGase in food processing, biopharmaceutical and other industrial processes can be directly improved.

CHAPTER 1
LITERATURE REVIEW

1.1 Activation mechanism of glutamine transaminase

Since 1989, when Ando et al. screened Streptomyces mobaraensis, a microorganism capable of secreting glutamine transaminase (TGase) from soil, the production of TGase by microbial fermentation has become the main method in industry. In this process, S. mobaraensis first secretes pro-TGase in the form of zymogen. There is a 45-amino acid protgase region in the n-terminal of S. Mobaraensis, which not only inhibits the activity of the enzyme, but also plays an important role in the efficient folding and secretion of the enzyme. The secreted pro-TGase first excites the first 41 amino acids of the proenzyme region by Transglutaminase activating metalloprotease (neutral protease) to produce a FRAP-TGase with certain activity. Then calcium ions depend on three peptidyl aminopeptidase (Tripeptidylaminopeptidase, TAP) FRAP further excision, the resulting TGase of maturity. Zhang's study showed that when EDTA, a neutral protease inhibitor, and PMSF, a TAP inhibitor, were added to the fermentation medium of S. mobaraensis, only EDTA could inhibit the increase of TGase activity, indicating that neutral protease played a key role in the activation of TGase.[7] Jang et al. found that TGase in Streptomyces hygroscopicus underwent a similar activation process. Schmidt et al. found that S. mobaraensis secreted a dual protease inhibitor, Streptomyces subtilisin and glutamine transaminase-activated protease inhibitor (Streptomyces subtilisin and neutral protease inhibitor). SSTI) - is the physiological substrate of TGase. They speculated that under physiological conditions, the activity of neutral protease is initially inhibited by SSTI, and when a small amount of pro-TGase is activated, it catalyzes the polymerization of SSTI to form an insoluble complex, thereby lifting the inhibition of SSTI on neutral protease, resulting in the increase of neutral protease activity, and then activating more pro-TGase. A positive feedback mechanism is formed. Long fatty chain amino acids similar to N-

lauroylsarcosine (LS) can block the glutamine site of SSTI and inhibit the polymerization of SSTI, thus controlling the activation process of TGase. These findings are critical to understanding the fermentation process of TGase in industrial production, and provide a theoretical basis for improving the fermentation production of TGase through genetic engineering or protein engineering. By controlling the activation process of TGase, the production efficiency and product quality of TGase can be improved, and then it can play a greater role in food processing, biomedicine, cosmetics and other fields.[4]

1.2 Activation of other proteases

In the production and application of glutamine aminotransferase (TGase), the activation of zymogen is a key step.[5] Dispase, bovine trypsin, chymotrypsin and other proteases can be used as activated proteases, and they can activate TGase by cutting at different sites in the TGase proenzyme region to remove the inhibitory region. Zotzel et al. purified a novel protease from the leach solution of Streptomyces mobaraensis mycelium, which can specifically activate glutamine transaminase prozyme.[7] Hence the name Transglutaminase activating metalloprotease (neutral protease). Juettne et al. successfully located the neutral protease gene in the genome of S. mobaraensis by homologous sequence alignment technique. [6]Compared with other proteases such as Dispase, bovine trypsin and chymotrypsin, neutral protease is highly specific to the cutting site of pro-TGase, and it only removes the prozyme region of TGase without degrading the active center of TGase, which makes neutral protease an ideal activation protease.[8] Neutral protease has no degradation effect on TGase, which ensures the integrity and activity of TGase in the activation process, which is of great significance for industrial production. Neutral protease is a protease that activates pro-TGase in wild bacteria, and its action mechanism and efficiency in nature provide an important reference for the industrial production of TGase. The discovery and characterization of neutral protease are of great significance for understanding and improving the production process of TGase.[9] Through the

activation of TGase by neutral protease, the yield and activity of TGase can be increased, the production cost can be reduced, and the efficiency of TGase in various applications can be improved. As a specific activated protease, neutral protease has broad application prospects in TGase production, food processing, biomedicine, cosmetics and other fields. In addition, the specificity and efficiency of neutral proteases provide new strategies for the activation of other enzymes.

1.3 Enzyme immobilization technology

Enzyme immobilization is a method of confining or binding an enzyme to a support in order to improve its stability and reusability. This technology is particularly important for industrial applications, as it can significantly reduce costs, increase production efficiency, and enable enzymes to operate under extreme conditions.[10] The following are several commonly used enzyme immobilization techniques.

1.3.1 Enzyme immobilization technology by adsorption

The enzyme molecules are fixed on the surface of the carrier by electrostatic adsorption or ion exchange without the formation of covalent bonds. The immobilization process is carried out under mild conditions and usually does not involve extreme pH or temperature, helping to keep the enzyme active.[11] Since adsorption is reversible, enzyme molecules can be desorbed by changing conditions, such as pH or ionic strength.[1] Due to the mild immobilization conditions, the enzyme is not easily denatured during the adsorption process, so it is usually able to maintain a high activity. Diatomaceous earth, mesoporous molecular sieve, ultrafine silicon oxide and other materials are commonly used as adsorption carriers because of their high specific surface area and good mechanical strength. Due to the relatively weak adsorption force, the immobilized enzyme may gradually fall off the carrier during the reaction, resulting in poor reusability. The immobilized enzyme by adsorption

method has been widely used in food processing, drug synthesis and environmental treatment because of its simple operation, mild condition and high recovery rate.

1.3.2 Embedding enzyme immobilization technology

Immobilization by embedding method is a method of immobilization of enzymes confined to porous or reticular materials. This technique works by physically restricting the movement of enzyme molecules while allowing small molecule substrates and products to freely diffuse between the enzyme and gel network. The enzymes are confined to gels with porous or reticular structures, rather than bound by chemical bonds. The substrates and products of small molecules can diffuse freely to the active site of the enzyme. [13]The embedding process is usually carried out under mild conditions and helps to maintain enzyme activity. Since enzymes are confined to the inside of the gel, there may be diffusion limitations of substrates and products. In some cases, enzymes may leak out of the gel structure. Compared with adsorption method and covalent crosslinking method, the stability of immobilized enzyme by embedding method may be lower. Macromolecular materials that can form gels, such as sodium alginate, polyacrylamide and gelatin, are often used for embedding immobilized enzymes.

1.3.3 Covalent cross-linking enzyme immobilization technology

Covalent cross-linking immobilized enzyme technology is a method to bind enzyme molecules to carriers through chemical covalent bonding. This technique utilizes bifunctional agents, such as glutaraldehyde, to form stable covalent bonds on the surface of the carrier and the enzyme molecule, thus achieving enzyme immobilization. The binding force of the covalent bond is strong, which makes the immobilized enzyme have high stability under various operating conditions. Due to the stability of the covalent bond, the immobilized enzyme is not easy to fall off the carrier, so the risk of leakage is low. During immobilization, some enzyme activity may be lost due to covalent modification. A variety of groups can be activated and

covalently react with amino acid residues on proteins, such as the ε-amino group of lysine residues. Chitosan and glutaraldehyde are commonly used as immobilized carriers and bifunctional reagents.[14] Chitosan is a kind of biomacromolecule composed of aminosaccharides, which can be obtained by deacetylation of chitin and is abundant in resources. Glutaraldehyde molecules have an aldehyde group at each end of the carbon chain, and can cross-link with chitosan and amino groups on enzyme molecules. [15]Covalent cross-linking immobilized enzymes have been widely used in biocatalysts, biosensors, drug delivery systems and other fields.

1.3.4 Other enzyme immobilization techniques

The development of enzyme immobilization technology continues to incorporate new materials and ideas to improve the performance of immobilized enzymes, including their stability, reusable ability and catalytic efficiency. In addition to the three basic technologies of adsorption, embedding and covalent crosslinking, there are a variety of improvements and composite applications. The following are some other enzyme immobilization techniques: Cross-linked encapsulation: Xia Yupei et al. used cross-linked encapsulation to encapsulate enzyme proteins by forming a cage graft structure on the surface of the carrier, in order to reduce mass transfer resistance and improve the reusable ability of the enzyme.[16] Composite materials: Yao Shanshan et al. prepared composite materials with both mechanical strength and reactivity by introducing active chitosan materials on the surface of silica with high mechanical strength. Nanomaterials: With the development of nanotechnology, nanomaterials such as carbon nanotubes, graphene derivatives, and metal nanoparticles have been used for enzyme immobilization due to their unique physical and chemical properties. These materials can provide more active sites and enhance the stability and catalytic efficiency of enzymes.[17] Layered complex: With layered complex technology, multiple functions can be introduced at different levels, for example, by constructing multiple layers of enzymes or other proteins on the surface of the carrier through layer self-assembly technology to improve the loading capacity

and catalytic efficiency of the immobilized enzyme. Bioaffinity immobilization: The use of bioaffinity, such as ligand-receptor interactions, to fix an enzyme to a specific carrier is often very beneficial for maintaining the natural conformation and activity of the enzyme.[18]

These new immobilized enzyme technologies show great potential for applications in biocatalysis, sensors, drug delivery, environmental protection and energy production.

1.4 Introduction to glutamine transaminase

Glutamine transaminase (TGase), also known as Transglutaminase, is a widely existing enzyme ina variety of organisms, its main function is to catalyze the covalent cross-linking of proteins within or between molecules. TGase can connect the γ-carboxyamide group of glutamine residue in protein with the ε-amino group of lysine residue through acyl transfer reaction to form the ε-(γ-glutamyl) lysine isopeptide bond.[19]

TGase is particularly important in the food industry because it can improve the texture of food, increase the nutritional value of proteins, form heat and water resistant films, and improve the elasticity and water holding capacity of food. In addition, TGase has potential applications in the field of medicine, such as polymerizing collagen in tissue engineering to produce hydrogels and scaffolds, and playing a role in the treatment of celiac disease and food allergies. The thermal stability and catalytic activity of TGase are its key characteristics in industrial applications.[20] Previous studies have significantly improved the thermal stability and catalytic activity of TGase through rational design and molecular modification methods, which will help expand the application of this enzyme in food and emerging fields.[21] In addition, the sources of TGase are diverse, including animals, plants and microorganisms, among which microbial TGase has a wide application prospect in industry because of its easy availability and mild reaction conditions. Other applications of TGase include improving the texture of meat, fish and dairy products,

increasing the volume of baked goods, improving the texture and extending the storage life of tofu, and as a mineral absorption accelerator. In general, as a multifunctional enzyme, TGase has a wide range of applications and development prospects in food processing, biomedicine and other industrial fields.[22]

1.5 Application of glutamine transaminase

TGase is used to improve the texture of food products, such as improving the elasticity and texture of meat products, enhancing the stability and taste of dairy products, and improving the texture of soy products. It can replace traditional additives such as phosphate for the production of low-salt meat products. In the biomedical field, TGase can be used to polymerize collagen in tissue engineering to produce hydrogels and scaffolds, as well as play a role in the treatment of celiac disease and food allergies.[23] TGase can be used in the cosmetics industry to improve the performance of products, such as improving the stability of proteins in cosmetics. In the textile industry, TGase can be used to improve the feel and durability of fabrics. TGase can catalyze covalent cross-linking between proteins and small molecules, and is used in protein modification, such as in the research and development of novel protein products. TGase can be used in the preparation of microcapsules, which is particularly important in drug delivery systems. TGase is also used in the preparation of edible films, which can be used in food packaging to increase the shelf life of food. TGase can be used to prepare emulsion gels and improve the stability of emulsions. TGase can covalently cross-link essential amino acids such as lysine to proteins and improve the nutritional value of proteins. TGase can be used to prepare solid fat substitutes with good texture and flavor.[24] TGase can be used as a mineral absorption promoter to improve the body's absorption of certain minerals. TGase is also used in the preparation of protein gels to improve their strength. TGase can be used to increase the viscosity of sticky rice and maintain its original taste and texture in storage. TGase can be used to increase the viscosity of milk. TGase is used to prevent the softening of brittle desserts. These applications of TGase show its importance as a

multifunctional enzyme in modern industry. With a better understanding of the catalytic mechanism of TGase, its application areas may be further expanded.[25]

1.6 Source of glutamine transaminase

TGase is widely found in natural organisms. Since the first discovery of TGase by Clarke et al. 7 in guinea pig liver in 1957, researchers have successively found TGase in fish, black wood and other animals as well as microorganisms and plant organisms. Guinea pig transglutamimase (GTG) from the liver of guinea pigs was the only source of commercial TGase for nearly 30 years, beginning in the 1960s. Due to the scarce source of enzymes and the complex separation and purification process, the price of enzymes is very high.[24] In the 1990s, European countries extracted coagulation factor X(a type of TGase) from the blood of slaughtered cattle and pigs for commercial application, but because the TGase extracted from the blood requires thrombin activation, it will produce red pigment deposition, affecting the appearance of the product. In 1987,cekson and Apelbaum discovered the activity of TGase in peas.[25] Subsequently, researchers have found the presence of this enzyme in a variety of plant tissues (cytoplasm, cell wall, chloroplast and mitochondria), such as Jerusalem artichoke, potato and corn, but the role of TGase in plant tissues remains unclear. Kang et al. have studied the extraction of TGase from soybean leaves, but the separation and purification process is complicated and the enzyme yield is low. Microbial transglutami-nase (MTG) is mainly derived from Streptomycesspp. And Bacillusspp. At present, there is no plant-derived TGase used for commercial production of microbial TGase(MTG). [26]In 1989,Ando et al. isolated microbial derived TGase from Streptomyces mobaraensis for the first time. The enzyme has a molecular weight of 37.9kD and is composed of 331 amino acids. The active center contains cysteine residues, and the activity is independent of calcium ions.[27] Subsequently, researchers found the existence of TGase in different strains of S. mumaraensis, and carried out a large number of related studies, such as fermentation process optimization, enzyme purification, gene engineering expression, etc., and

made gratifying progress. Although a variety of microorganisms can produce TGase by fermentation, only the TGase derived from S.mobaraensis has been commercialized to produce microbial TGase, which is an extracellular enzyme and can be directly secreted into the medium. It is easier to separate and purify TGase from animal and plant sources, and the microbial fermentation raw materials are cheap and the enzyme production cycle is short, which is the most promising for large-scale industrial production, so it is favored by researchers 2)].[28] In recent years, microbial sources of TGase are gradually replacing animal sources of TGase for commercial production, becoming the main commercial source of TGase since 1989, the international research using microbial fermentation to industrialize the production of TGase. Since the food industry TGase put on the market in 1993, the development is very rapid, the scope of application continues to expand, it is reported that in 1995, the sales of the enzyme preparation of nearly 2 billion yuan, in 2000 TGase has become Japan's second largest industrial enzyme.[29] China in July 1, 2001, TGase as a new supplement, included in the ranks of food additives, but China has dozens of universities and scientific research institutions (including enterprise research institute) in TGase production, development, application research, and has achieved a series of results, there are a number of well-known domestic enterprises in the use of TGase.

Conclusions to chapter 1

At present, with the further improvement of our people's material life, people are paying more and more attention to the quality, flavor and safety of food, and high-quality meat products, fish products, dairy products, soy products, flour products and low-salt, low-fat health meat products are becoming more and more popular. The combination of proteins from different sources of animals and plants is very much in line with Chinese tastes. These are the areas where TGase plays its role.[30]

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

The objectives of the study of the research in this field aims to explore the potential effects of different types of proteases on TGase activity, as well as the molecular mechanisms of these effects. In addition, the study aims to determine which conditions maximize the activity and stability of TGase, thereby providing guidance for the application of TGase in various industrial processes.

Strains – *Streptomyces pentadiengensis* was preserved in the laboratoryt.

Alkaline protease, neutral protease, chymotrypsin, trypsin, papain, cultured by our laboratory staff in previous studies.

2.1 Reagents and instruments

Main reagents

α-N-CBZ-Gln-Gly, L-glutamate-γ-hydroxamic acid, ssDNA were purchased from Sigma-Aldrich Company (Shanghai). Other reagents used in this study were purchased from Sinopharm Group Chemical Reagent Co., LTD., and the purity was analytical pure.

Main instruments

The main instruments used in this study are listed in Tab. 2.1 below.

Medium

LB medium (g/L) : Tryptone 10, yeast extract 5, sodium chloride 10.

TB medium (g/L) : Tryptone 12, yeast extract 24, potassium dihydrogen phosphate 2.31, dipotassium diphosphate trihydrate 16.37, glycerol 5.

Seed medium (g/L) : Glycerin 20, peptone 20, yeast powder 5, magnesium sulfate heptahydrate 2, dipotassium hydrogen phosphate 2, potassium dihydrogen phosphate 2, pH7.0

Fermentation medium (g/L) : glycerin 20, peptone 20, yeast powder 5, cornmeal 20, dipotassium hydrogen phosphate 4, potassium dihydrogen phosphate 2, magnesium sulfate heptahydrate 2, ammonium chloride 3.2

Table 2.1 – Main instruments used in this chapter

№	Device name	Manufacturer
1	Model 5418R centrifuge	Eppendorf GMBH
2	Model H1850 centrifuge	cence
3	J6-MI type centrifuge	Beckman Coulter Inc
4	Allegra X-15R centrifuge	Beckman Coulter Inc
5	ZQZY-CF8 type shaker	Shanghai Zhchu Instrument Co., LTD
6	DK-8D type three-hole three-temperature water bath	Changzhou Aihua Instrument manufacturing Co., LTD
7	ST3100 pH meter	Ohaus Instrument (Changzhou) Co., LTD
8	Analytical balance (accurate to 0.0001g)	METTLER TOLEDO GMBH
9	Electronic balance (accurate to 0.01g)	Ohaus Instrument (Changzhou) Co., LTD
10	5 L fermenter	Shanghai Dipir Co., LTD

2.2 Detection of glutamine transaminase activity

Detection of glutamine transaminase activity is essential to evaluate its efficacy in biochemical processes and industrial applications. In this experiment, we used colorimetry to determine the activity of TGase, and used specific substrates and standard curves to quantify the enzyme activity. Colorimetry is a commonly used method for the determination of enzyme activity, which depends on the light absorption properties of the products of enzyme-catalyzed reactions. In this experiment, α-N-CBZ-Gln-Gly, as a substrate, was catalyzed by TGase to produce L-glutamate-γ-monohydroxylamine, the amount of which could be measured by the

increase of light absorption. The activity of 1 U TGase is defined as the amount of enzyme (U/mL) that catalyzes the formation of 1 μmol L-glutamate-γ-monohydroxylamine at 37 ° C per minute. At 37℃, the reaction was allowed to continue for 10 minutes. Before adding the substrate, in order to activate glutamine transaminase, a neutral protease with a final concentration of 5 μg/mL was added and activated at 37℃ for 20 minutes. The activation buffer consists of Tris 50 mM, NaCl 300 mM, CaCl2 2 mM, GSH 1 mM, and pH adjusted to 7.0 with hydrochloric acid. Neutral protease should be diluted to 100 μg/mL before use. A series of standard solutions with known concentrations of L-glutamate-gamma-monohydroxylamine are prepared and a standard curve is drawn by measuring their absorbance at a specific wavelength. The activated glutamine transaminase and α-N-CBZ-Gln-Gly substrate were added to the reaction system, and the amount of L-glutamate-γ-monohydroxylamine was determined by colorimetry after the reaction at 37℃ for 10 minutes. According to the change of absorbance, the standard curve was used to calculate the amount of L-glutamine-gamma-monohydroxylamine catalyzed by glutamine transaminase, so as to calculate the activity of TGase. Colorimetry provides a rapid and accurate method for the determination of TGase activity. The experimental results show that the activity of glutamine transaminase can be measured and improved effectively through specific activation steps and reaction conditions.

2.3 Detection of protease activity

Spectrophotometry is a commonly used method to determine enzyme activity. It quantifies enzyme activity by measuring the change of absorbance of reaction product or substrate. The activity of protease was determined by spectrophotometry. The protease catalyzes the hydrolysis of casein to produce tyrosine. The addition of trichloroacetic acid stops the reaction and precipitates the unreacted substrate. The filtrate containing tyrosine is obtained by filtration. Subsequently, sodium carbonate and Folin's reagent are added to form a blue complex of tyrosine, whose absorbance

is proportional to the concentration of tyrosine, so that the protease activity can be determined. Three 10 mL test tubes with plugs were taken, one as the sample blank tube and two as the sample tube. Accurately add 1.0 mL diluted enzyme solution to the three test tubes. Put the test tube in a 40℃±0.2℃ water bath and preheat it for 5 minutes. Add 1.0 mL preheated 1% casein solution to each of the two sample tubes. Accurate timing, let the reaction in 40℃±0.2℃ water bath for 10 minutes. 2 mL 0.4 mol/L trichloroacetic acid solution was quickly added to the three test tubes to stop the reaction, and 1.0 mL 1% casein solution was added to the blank tube of the sample. The test tube was placed in a 40 ° C ±0.2 ° C water bath for another 10 minutes. Remove the tube, cool quickly to room temperature, and strain with a medium-speed qualitative filter paper. Three 10 mL test tubes with plugs were also taken and 1.0 mL of the above filtrate was absorbed into them respectively. Add 5.0 mL 0.4 mol/L sodium carbonate solution and 1.0 mL dilute folin reagent into each of the three test tubes, shake well and put them in a water bath at 40 ° C ±0.2 ° C for 20 minutes. Remove the tube and place it in cold water to cool quickly to room temperature. The absorbance of sample tube and sample blank tube was determined with a 10 mm cuvette at 680 nm wavelength using a spectrophotometer. The average absorbance between the sample tube and the sample tube was calculated with the reagent blank tube zeroed, and the concentration of the produced tyrosine was obtained by the standard curve of tyrosine or the linear regression equation. Spectrophotometry provides an accurate and reliable method for the determination of protease activity. Through this experiment, we can obtain the activity data of protease under specific conditions, which is of great significance for further study of its biological functions and potential applications.

2.4 Improvement of glutamine transaminase activity by different proteases

Among the five selected enzymes, including alkaline protease, neutral protease and chymotrypsin, the enzyme with the highest activity was selected compared with the control group, and co-culture with glutamine transaminase promoted the

improvement of glutamine transaminase activity. In addition, the neutral protease has an efficient catalytic rate, which can rapidly hydrolyze protein peptide bonds. This enzyme works at neutral pH and is usually stable between pH 6.0 and 9.0, which makes it useful in a variety of applications, especially those requiring mild conditions in biotechnology processes. In addition, it has thermal stability and has good catalytic effect over a wide range of temperatures (10 degrees Celsius to 60 degrees Celsius). (As shown in Fig. 2.1)

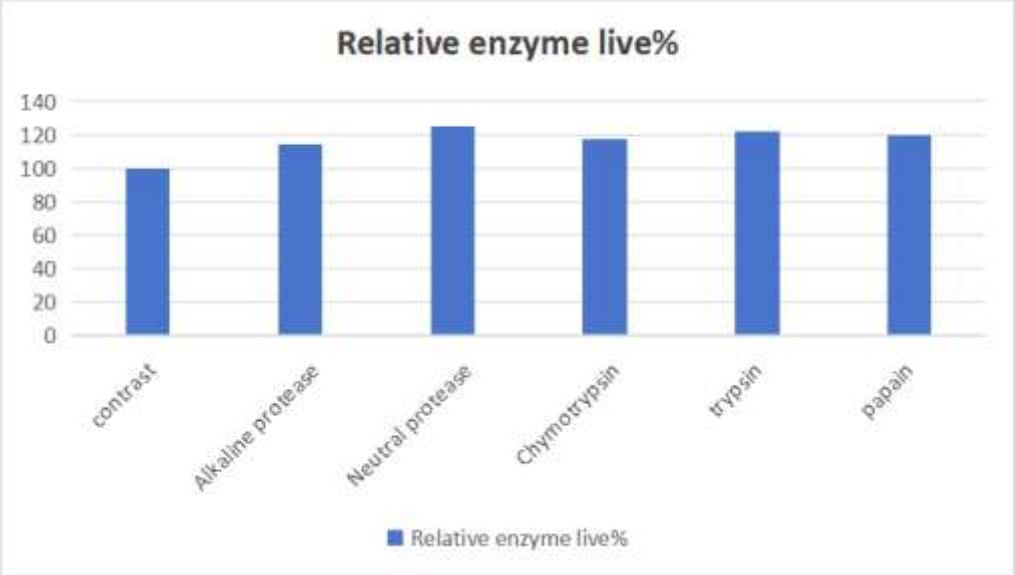

Figure 2.1 – Enzyme activity of the control group with five proteases

2.5 Preparation of chitosan pellets

The preparation of chitosan pellets is an application of nanotechnology or microcapsule technology. As a natural polysaccharide, chitosan has good biocompatibility and biodegradability, and is often used in the preparation of drug delivery systems and enzyme immobilization carriers. The following is a detailed description of the preparation process of chitosan pellets:

Dissolve chitosan in 1.5% (v/v) acetic acid solution until a 3% chitosan solution is formed. Acetic acid as a solvent is helpful for the dissolution of chitosan. Since bubbles may be created during the dissolution process, ultrasonic oscillations are used overnight to remove bubbles from the solution, ensuring that there are no bubbles during the pellet forming process. The chitosan solution was drawn with a 10

mL syringe and dropped drop by drop into a solution containing 1 M KOH and 25% (v/v) ethanol. The mixed solution of KOH and ethanol facilitates the formation of chitosan pellets. In the process of adding chitosan solution, keep stirring at low speed to promote the formation of small balls. When dribbling, adjust the height difference between the syringe needle and the liquid surface to control the shape and size of the ball. A large height difference causes the ball to be flattened to form a disc, while a small height difference may cause the ball to trail. After the pellets were formed, they were allowed to rest for 2 hours to fully cure the chitosan pellets. The chitosan pellets of the same size, about 2 mm in diameter, were removed and thoroughly washed with water until the washing solution was neutral to remove unreacted reagents and by-products. After washing, the chitosan pellets were stored in 20 mM K2HPO4-KH2PO4 buffer at 4℃ and pH=7.4 to maintain their stability. The prepared chitosan pellets can be used for immobilized enzyme, drug slow release, catalyst carrier, etc. Because of its biocompatibility and biodegradability, it has wide application potential in the field of biomedicine.

2.6 Method and optimization of immobilized neutral protease

Immobilized enzyme technology is a method to combine enzyme with carrier to improve its stability and reusability. In this experiment, the neutral protease (NP) was immobilized by using chitosan pellets as the carrier, and the immobilization conditions were optimized to obtain the best immobilization efficiency and enzyme activity. The chitosan pellets were placed in 2% glutaraldehyde solution and the surface groups were activated for 2 hours. Glutaraldehyde, as a crosslinking agent, can react with the amino group on the surface of chitosan pellets to activate the surface. After activation, the pellets are removed and thoroughly washed with water to remove unreacted glutaraldehyde and prevent it from interfering with subsequent reactions. The washed chitosan pellets were stored in a phosphate buffer at 4℃ and pH=7.4. The pellets were placed in a solution containing 3.54 μM neutral protease at 4℃ and cross-linked for 24 hours for enzyme immobilization. After the cross-linking,

wash the pellets with a pH=7.4 buffer to remove unbound enzyme molecules. The immobilized neutral protease pellets were again stored in a buffer solution at 4℃ and pH=7.4. The activity and stability of the immobilized enzyme under each condition were evaluated by changing the above parameters. The response surface method (RSM) or orthogonal experimental design can be used to systematically study the interaction of multiple variables. The optimized immobilized neutral protease can be widely used in biocatalysis, drug release, food processing and other fields.

2.6.1 Determination of enzymatic properties of immobilized neutral protease

The optimal reaction temperature of immobilized neutral protease was determined by taking 10 immobilized neutral protease pellets. A 0.5% casein substrate was prepared in 20 mM K2HPO4-KH2PO4 buffer (pH=7.4). In the range of 40~80℃, a series of different reaction temperatures are set. The immobilized enzyme pellets are added to a substrate solution preheated to a specific temperature. At each set temperature, the catalytic activity of the immobilized neutral protease was measured. With the maximum enzyme activity of 100%, the reaction relative enzyme activity at other temperatures was calculated. The optimum reaction temperature of neutral protease was determined by analyzing the enzyme activity data at different temperatures.

The optimal reaction pH of immobilized neutral protease was determined by taking 10 immobilized neutral protease pellets. Prepare 400 μL of 0.5% casein substrate and adjust pH from 5 to 10. At each set pH, the immobilized enzyme pellets are added to the preheated substrate solution. The catalytic activity of immobilized neutral protease was measured at each pH. With the highest enzyme activity of 100%, the relative enzyme activity of the reaction at other pH conditions was calculated.

The temperature stability of immobilized neutral protease was determined by taking 10 immobilized neutral protease pellets. Add 200 μL buffer with pH=7.4. The immobilized enzyme pellets were incubated at 50℃ and 60℃ for 10, 30 and 60

minutes, respectively. After incubation, the reaction system was quickly iced for 5 minutes to stop the reaction. Blot the buffer and prepare to measure enzyme activity. The remaining catalytic activity was determined. The relative enzyme activity under other incubation conditions was calculated with the unincubated enzyme activity being 100%.

2.6.2 Reuse detection of immobilized enzyme

The reusability of immobilized enzymes is an important index to evaluate their cost-effectiveness and practicality in industrial applications. Take 10 immobilized neutral protease pellets. At 37℃, the immobilized enzyme pellets were activated with 400 μL 10.84 μM pro-TGase for 10 minutes. The enzyme activity of the activated TGase was measured immediately after activation. SDS-PAGE analysis was performed to assess glutamine transaminase activation and any possible protein degradation. Wash the immobilized enzyme pellets with a pH 7.4 buffer to remove unreacted glutamine transaminase from the reaction mixture. After cleaning, re-add fresh glutamine transaminase and repeat the activation process. Glutamine transaminase activity was measured after each activation for 10 consecutive times, and SDS-PAGE analysis was performed after the last activation. The glutamine transaminase activity measured after each activation was compared to evaluate the reusability of immobilized neutral proteases. The high reusability of immobilized enzymes can significantly reduce the cost in industrial production and improve production efficiency.

CHAPTER 3
EXPERIMENTAL PART

3.1 Elevation of glutamine transaminase by different proteases

In the process of exploring to improve the activity of glutamine transaminase (TGase), we have carried out detailed screening experiments on five different proteases, such as alkaline protease, neutral protease and chymotrypsin. These experiments aim to identify which proteases are most effective in promoting TGase modification and maturation, thereby enhancing its biological function. TGase was co-cultured with five different proteases, and the concentration, pH value, temperature and other culture conditions of each protease were consistent to ensure the comparability of experiments. A control group without any proteases was set up to evaluate the specific effects of other proteases on TGase activity. The activity of TGase was measured at different time points in the culture cycle by standardized enzyme activity measurement methods. After data collection, the TGase activity of different protease treatment groups was compared with the control group using a bar chart. Among the five selected enzymes, including alkaline protease, neutral protease and chymotrypsin, the enzyme with the highest activity was selected compared with the control group, and co-culture with glutamine transaminase promoted the improvement of glutamine transaminase activity. It can be seen from the figure that among the above five proteases (as shown in Fig. 3.1), neutral protease had the highest enzyme activity compared with the control group, that is to say, neutral protease could promote the modification maturation of glutamine transaminase more than the other four proteases.

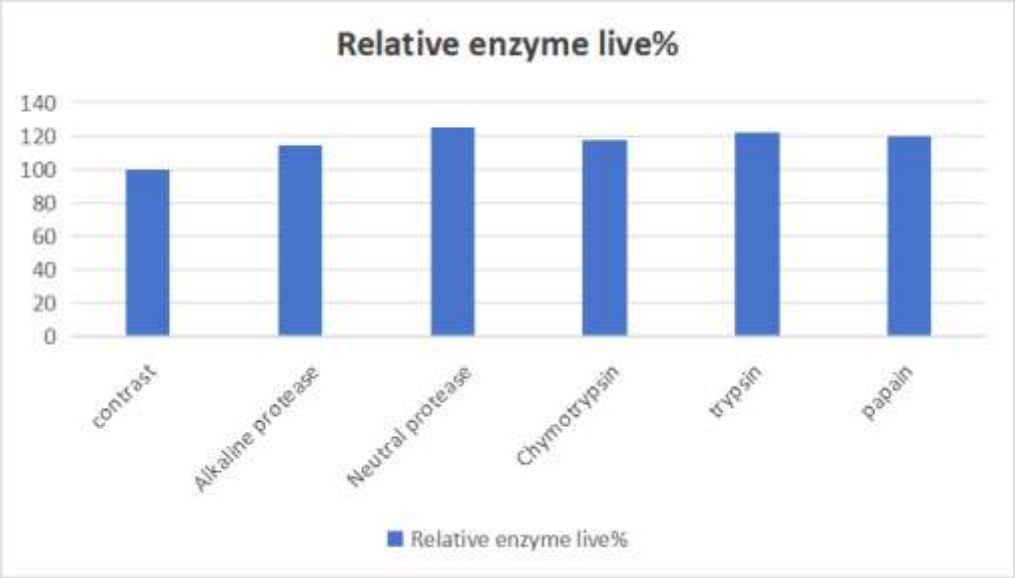

Figure 3.1 – Enzyme activity of the control group with five proteases

3.2 Influence of neutral protease on glutamine transaminase activity

In this experiment, we aimed to investigate the effect of neutral protease on glutamine transaminase (TGase) activity. The experiment was carried out by setting up two parallel experiments, in which the same basal amount of TGase was added to each experiment, and the only variable was whether neutral protease was added or not. The purpose of the experiment was to reveal whether neutral protease can be used as an effective modifier to enhance the activity of TGase. Two groups of experiments were set, equal amount of glutamine transaminase was added, and the variable was set as whether neutral protease was added or not. The experimental results were recorded every eight hours, and the following chart was made (as shown in Fig. 3.2). It can be seen from the bar chart that the enzyme activity in both groups was low before 32 hours, because the enzyme was not fully activated after too short a time. After 32 hours, the enzyme activity of the two groups gradually stabilized, and it could be observed that the activity of the group with neutral protease was significantly higher than that of the group without neutral protease. The enzyme activity of the group with neutral protease was stable at 7 U/mL, while that of the group without neutral protease was only 4 U/mL, indicating that neutral protease had a significant promoting effect on the activity of glutamine transaminase. The

experimental results showed that, The neutral protease can significantly promote the activity of TGase, and the activity is increased by about 75%.

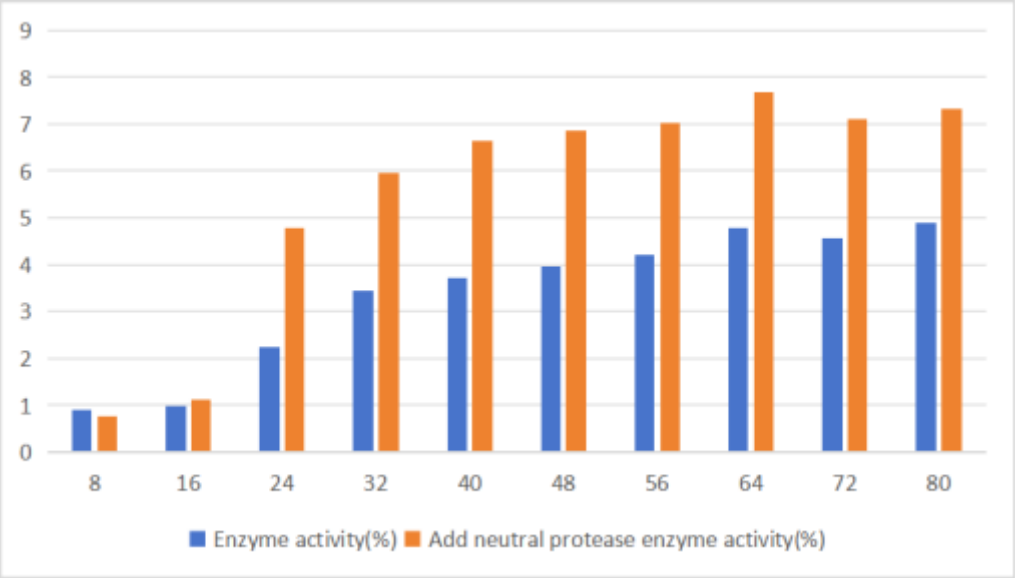

Figure 3.2 – Normal enzyme activity of TGase and enzyme activity of neutral protease

3.3 Study on the enzymatic properties of neutral proteases

3.3.1 Effect of pH on neutral protease activity

In an in-depth study of how the activity of neutral proteases is affected by pH, we measured changes in enzyme activity in detail by systematically changing the pH environment from acidic to alkaline. This experiment not only helps to reveal the working efficiency of neutral protease under different pH conditions, but also provides a theoretical basis for the optimization of conditions in practical applications. A neutral protease solution with a certain concentration was prepared under laboratory conditions. The neutral protease solution was adjusted to pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, and pH 10.0 for a total of six different pH values to cover a wide range from strongly acidic to strongly alkaline. At each set pH, a standardized enzyme activity assay was used to measure the activity of neutral proteases. The enzyme activity measured at each pH value was recorded for subsequent analysis (see Figure 3.3).

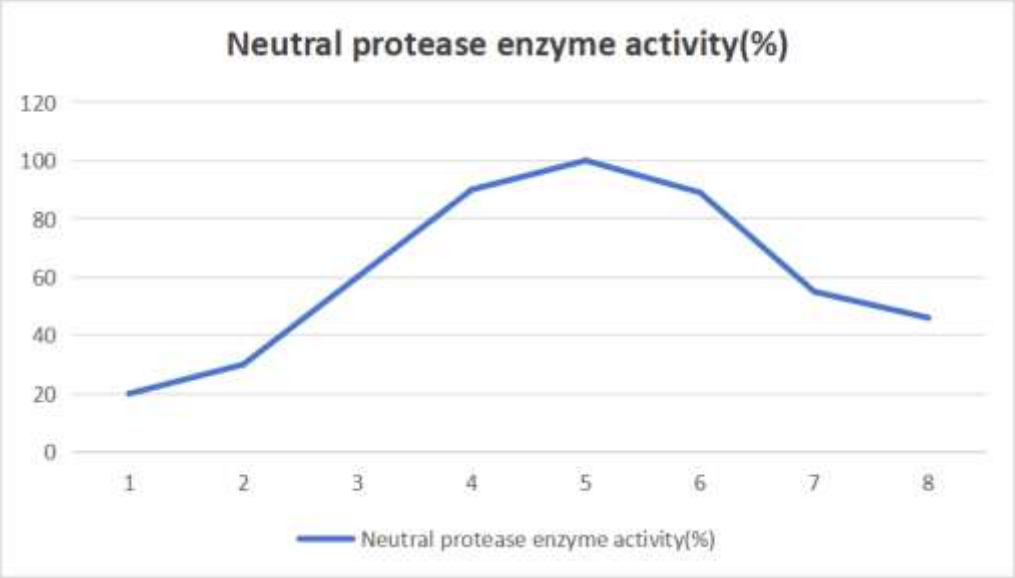

Figure 3.3 – Effect of pH on neutral protease activity

Through the line chart, we can intuitively see the relationship between pH and neutral protease activity. At pH 5.0, the enzyme activity decreased significantly, indicating that the strong acidic environment inhibited the activity of neutral protease. When the pH value is adjusted to 7.0, the activity of the neutral protease reaches its highest point, close to 100% relative activity, indicating that this is the best working pH value of the neutral protease. Even in the weakly alkaline environment of pH 8.0, the activity of neutral protease can be maintained at a high level, more than 90%, showing its stability and activity under near-neutral to weakly alkaline conditions. However, in the strongly alkaline environment of pH 10.0, the enzyme activity again decreased, which may be due to the change in the molecular structure of the enzyme caused by the high pH value. Neutral proteases exhibit high activity in neutral to weakly alkaline environments, with an optimal pH operating range of 6.5 to 8.0. This finding has important practical significance for guiding the use of neutral protease in industrial production, such as detergent addition, food processing, leather treatment, etc.

3.3.2 Influence of temperature on the activity of neutral protease

In order to deeply understand the effect of temperature change on neutral protease activity, we designed a series of experiments and drew a line graph of the relationship between temperature and activity. Determine the optimal temperature range for neutral proteases and understand the specific effects of temperature changes on enzyme activity (Fig. 3.4).

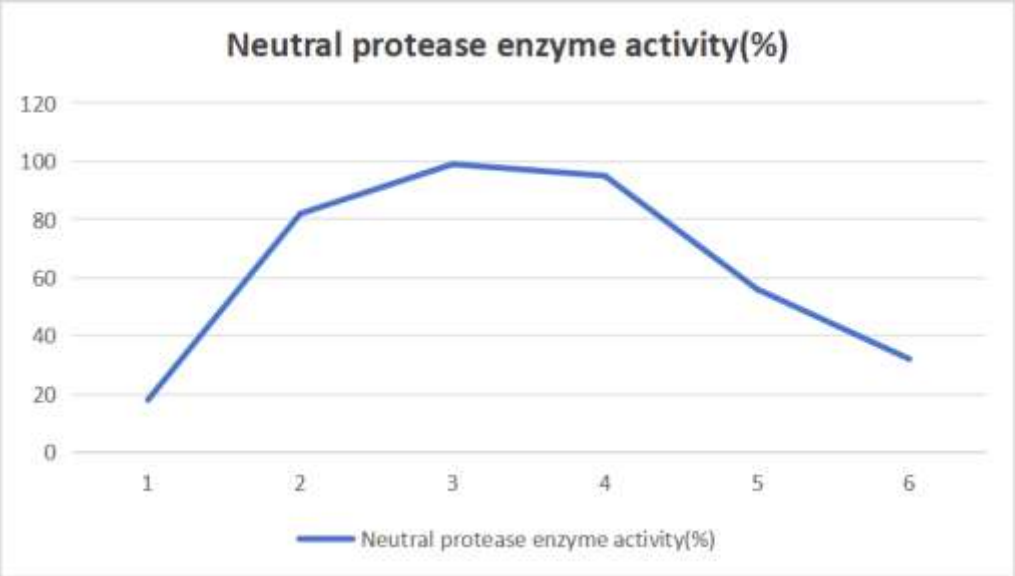

Figure 3.4 – Effect of temperature on the activity of neutral protease

In the range of 20 ° C to 80 ° C, a temperature point is set every 10 ° C, a total of 7 different temperature conditions. At each temperature point, the neutral protease solution is adjusted to the corresponding temperature and maintained for a period of time to achieve thermal equilibrium. At each temperature point, a standardized enzyme activity assay was used to measure the activity of neutral proteases. Enzyme activity at each temperature point was recorded, and a line chart was used to show the relationship between activity and temperature. At 20℃, the activity of neutral protease is the lowest, which may be due to the lower temperature limits the thermal movement of enzyme molecules, resulting in a lower frequency of substrate and enzyme collision, thus affecting the catalytic efficiency of the enzyme. From 20℃ to 50℃, the activity of neutral protease increased steadily with the gradual increase of temperature, indicating that the increase of temperature helps to improve the

flexibility and reaction rate of enzyme molecules, and thus improve the catalytic efficiency of enzyme. At 55℃, the activity of neutral protease reached the highest point, close to 100% relative activity, indicating that this temperature is the best working temperature of neutral protease. When the temperature continued to rise to more than 60℃, the enzyme activity began to decline, which may be due to the high temperature caused the enzyme protein began to denature, the active center structure was destroyed, thus reducing the activity of the enzyme. According to the trend of the line chart, the neutral protease has the highest activity between 50 ° C and 60 ° C and can play the most role.

3.3.3 Influence of incubation pH on neutral protease activity

In an in-depth study of the relationship between neutral protease activity and incubation pH, we used a series of experimental designs to accurately determine the effect of different pH values on neutral protease activity. The activity changes of neutral protease under different pH values were determined, and the optimal pH range was determined. In the pH 5 to pH 10 range, set up six experimental groups, each with the same pH interval, to ensure that the full range of strong acids to weak bases is covered (Fig. 3.5). At each set pH value, the neutral protease solution is adjusted to the corresponding pH value, and a certain incubation time is maintained to ensure that the enzyme molecules are fully adapted to the environment. The neutral protease activity at each pH was determined by using standardized enzyme activity assay method. The neutral protease activity at each pH was determined by using standardized enzyme activity assay method. In the strong acid environment of pH 5, the activity of neutral protease is very low, close to 0, indicating that the strong acid environment has a significant inhibitory effect on the activity of neutral protease. In the range of pH 6 to pH 10, the activity of the neutral protease was higher than 70%, indicating that the neutral protease was able to maintain high activity at these pH conditions. Between pH 7 and pH 9, the activity of neutral protease is the highest, especially at pH 8.0, the activity is close to 100%, indicating that neutral protease can

exert the greatest catalytic efficiency at this pH value. It can be intuitively seen from the line chart that the activity of neutral protease increases with the increase of pH value, reaching a peak at about pH 8, and then slightly decreasing with the further increase of pH value. Therefore, neutral proteases have the highest activity in an environment near neutral to weakly alkaline, especially at pH 8 or so.

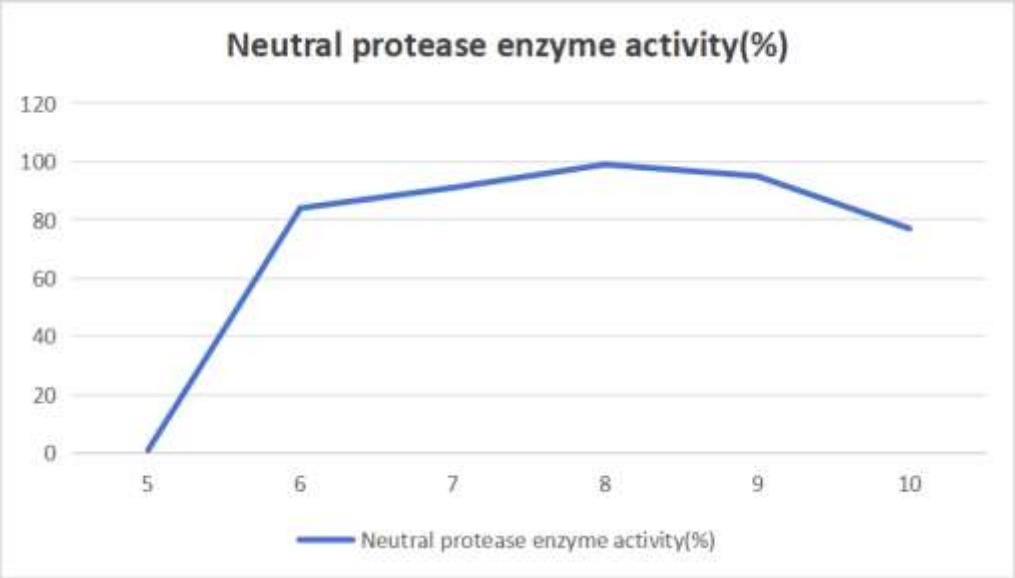

Figure 3.5– Effect of incubation pH on the activity of neutral protease

3.3.4 Influence of incubation temperature on neutral protease activity

In this study, we aimed to gain a deeper understanding of the effect of incubation temperature on the activity of neutral proteases, which is essential to optimize the conditions for this enzyme in industrial applications. By setting up a series of experiments at different temperatures, we were able to accurately assess the specific effects of temperature changes on neutral protease activity and determine its optimal incubation temperature. The effect of different incubation temperature on the activity of neutral protease was studied, and the optimum incubation temperature of neutral protease was determined. Incubation temperature points of 30℃, 40℃, 50℃ and 60℃ were selected to cover the possible suitable temperature range of neutral protease. Before the experiment, the neutral protease activity of all groups was adjusted to 100% to eliminate the influence of differences in initial activity on the experimental results. The activity of neutral protease was measured every 10 minutes

from 0 minutes to 60 minutes, and a total of 7 groups of data were recorded. Standardized enzyme activity assay methods are used to accurately measure enzyme activity at each time point. Enzyme activity data of each temperature point at different times were recorded in detail, and the change of activity with time and temperature was shown by line chart (Fig. 3.6).

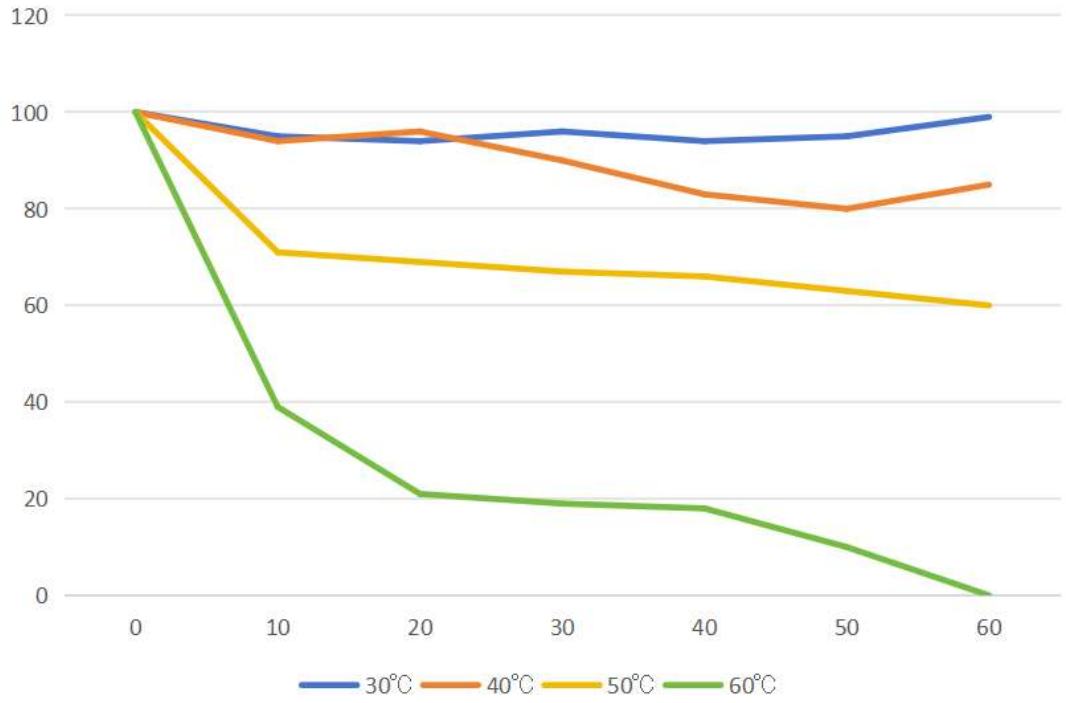

Figure 3.6 – Effect of temperature on the activity of neutral protease

High temperature group (60℃) : Within the first 20 minutes, the neutral protease activity rapidly decreased to 20%, and eventually completely deactivated. These results indicate that the structure of neutral protease can be destroyed rapidly in high temperature environment, resulting in denaturation and inactivation.

In the higher temperature group (50 ° C), the neutral protease activity rapidly decreased to about 70% in the first 10 minutes and continued to decline to 60% in the following 50 minutes. This shows that although 50℃ is lower than 60℃, it still has a significant inhibitory effect on the activity of neutral protease.

Moderate temperature group (40℃) : the activity decreased less, remained at about 85%, but the final activity (88%) was slightly lower than that of the 30℃ group. This indicates that although 40℃ is suitable, it still has a certain adverse effect on the maintenance of enzyme activity.

In the low temperature group (30℃), the activity remained the highest, always around 95%, and the activity was 97% at the end of the experiment. The results showed that 30℃ was the best incubation temperature for neutral protease activity.

The activity of neutral protease was the highest at 30℃, and the maintenance time was the longest, which indicated that 30℃ was the best incubation temperature for neutral protease. In contrast, high temperatures (such as 60 ° C and 50 ° C) can lead to rapid inactivation of neutral proteases, while 40 ° C is more appropriate, but the activity is still not as good as 30 ° C.

3.4 Neutral proteases catalyze glutamine transaminase activation

In biotechnology and industrial applications, the immobilization of neutral proteases is an effective means to improve their stability and reuse. In this study, neutral protease was immobilized and compared with free neutral protease to evaluate the effect of immobilization on enzymatic properties. The effects of temperature and pH value on the activity of immobilized neutral protease and free neutral protease were compared. After incubation at 50℃ and 60℃ for 60 minutes, the active residues of the two forms of neutral proteases were measured. Incubated at pH 5 and pH 10 for 60 minutes, the active residues of the two forms of neutral proteases were measured. The optimum reaction pH of neutral protease before and after immobilization was determined by measuring the activity at different pH values. After immobilization, the sensitivity of neutral protease to temperature did not change significantly, but the thermal stability was enhanced. After incubation at 50℃ and 60℃ for 60 minutes, the active residues of free neutral protease were 57.3% and 0, respectively, while the active residues of immobilized protease increased to 83.3% and 19.3%, respectively. Immobilization results in the change of pH of neutral protease from 7 to 9, which may be related to the change of molecular conformation during immobilization. Immobilization significantly improved the tolerance of neutral protease to acid and base. After incubation at pH 5 for 60 minutes, the activity of free neutral protease was only 2.8%, while the activity of immobilized protease

remained 42.5%. After incubation at pH 10 for 60 minutes, the immobilized neutral protease activity remained 90.0%, while the free form activity remained 76.3%. Immobilization may enhance the conformational stability of the enzyme molecule by forming multiple covalent bonds between the enzyme molecule and the carrier, thereby improving thermal stability and pH stability. Immobilization treatment significantly improved the thermal stability and pH stability of the neutral protease, and changed its optimal reaction pH. (Fig. 3.7– 3.10)/

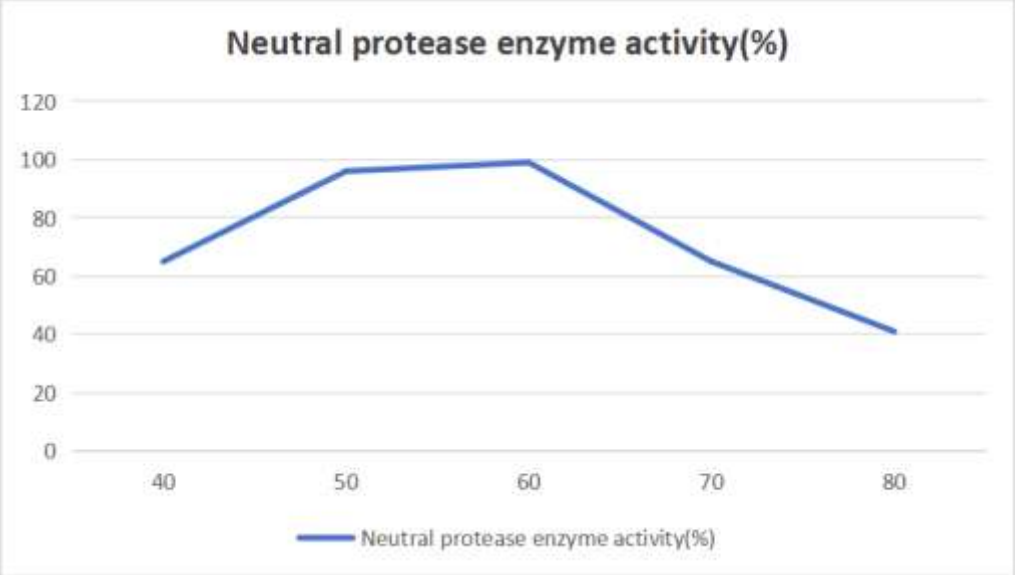

Figure 3.7 – Effect of incubation temperature on the activity of neutral protease

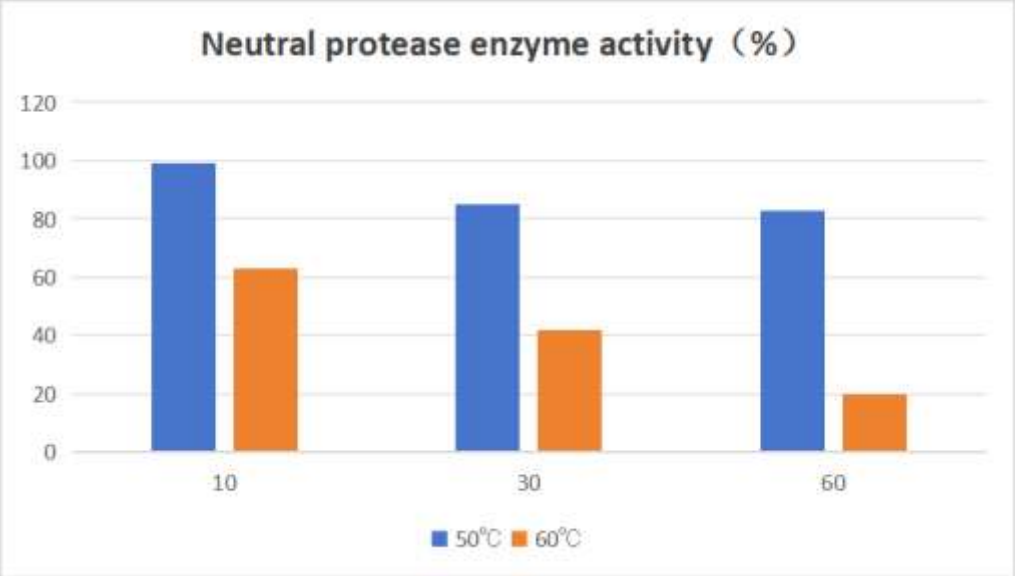

Figure 3.8 – Effect of incubation temperature on the activity of neutral protease

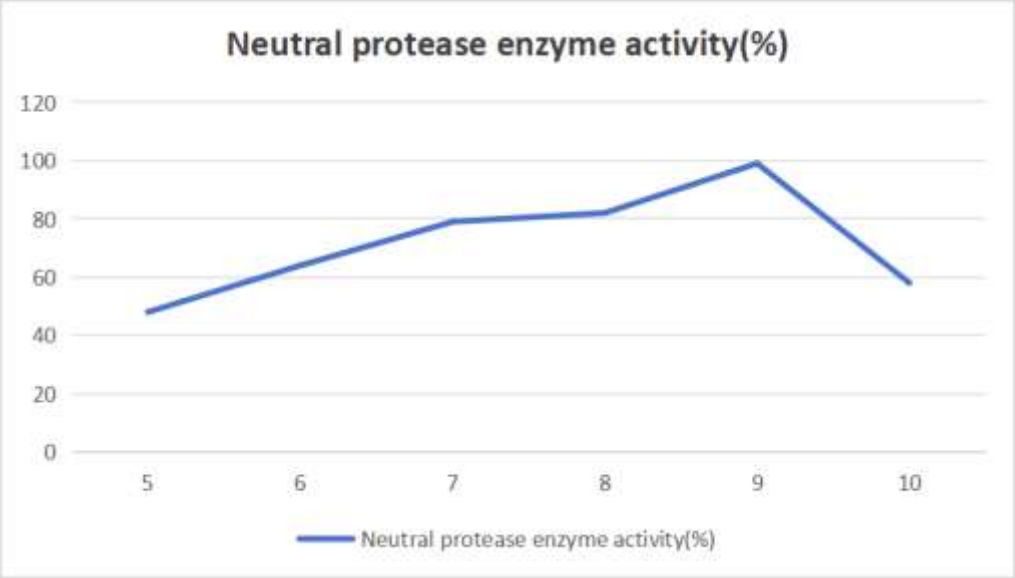

Figure 3.9 – Effect of incubation pH on the activity of neutral protease

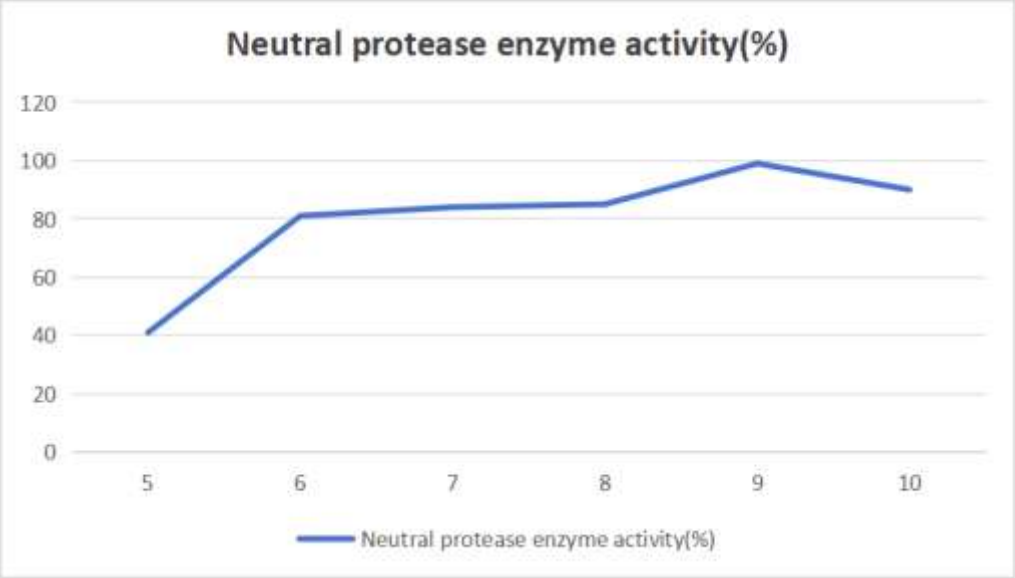

Figure 3.10 – Effect of incubation pH on the activity of neutral protease

3.5 Reusability analysis of immobilized neutral protease

In biocatalysis and industrial applications, the reusable performance of immobilized enzymes is an important consideration, as it directly affects production costs and ease of operation. The aim of this study was to evaluate the ability of immobilized neutral proteases to activate glutamine transaminase during continuous reuse to determine their potential value as industrial catalysts. Ten immobilized neutral proteases were used as experimental materials. 400 μL TGase solution with a

concentration of 10.84 μM was added to the immobilized enzyme each time. The activation reaction was performed at 37℃ for 10 minutes. After the reaction, the immobilized enzyme is washed with a pH 7.4 buffer to remove reaction products and unreacted substrates. Add the glutamine transaminase solution again and repeat the above activation and washing steps for a total of 10 cycles (Fig. 311). After each cycle, the glutamine transaminase activity produced by the activation is analyzed and compared with the activity produced by the first cycle. The activity changes and protein patterns of immobilized enzymes were analyzed using gel electrophoresis to assess their structural stability. During the first 6 reuses, the activation efficiency gradually decreased, which may be due to the gradual loss or inactivation of the active site on the surface of the immobilized enzyme during the initial use. From the 6th to the 10th reaction, the activation efficiency tended to be stable and remained at the activity level of about 75%, indicating that the immobilized enzyme could maintain a stable activation efficiency after the initial decline in activity. The electrophoretic results showed the same trend as the activity analysis, that is, the protein pattern and the active site of the immobilized enzyme remained relatively stable in repeated use. These results indicate that the immobilized neutral protease has good reuse performance and can stably activate TGase in multiple continuous uses. The immobilized neutral protease shows good stability and activation efficiency in repeated use, and can be used as an effective industrial catalyst for the activation of glutamine transaminase. Although there was a certain decline in activity at the initial stage, the activity tended to be stable at the later stage, indicating that the immobilized enzyme has adaptability and can achieve cost-effective and convenient operation in practical applications.

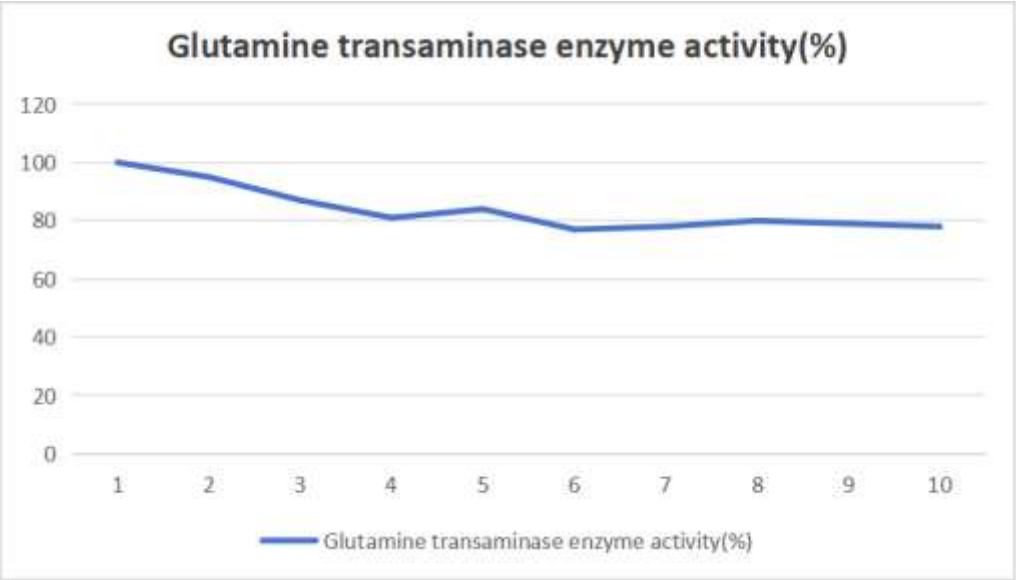

Figure 3.11 – Effect of times of use on glutamine transaminase activity

CONCLUSIONS

In this study, the effects of different proteases on the activity of glutamine aminotransferase (TGase), the promotion effect of neutral proteases on the activity of TGase, and the enzymatic properties and reuse performance of immobilized neutral proteases were investigated through a series of experiments. Here is a summary of the results:

1. Promoting effect of neutral protease on glutamine transaminase activity: During the 32-hour culture cycle, the activity of TGase with neutral protease was stable at 7 U/mL, while that in the control group without neutral protease was only 4 U/mL, indicating that neutral protease can effectively enhance the activity of TGase.
2. Enzymatic properties of immobilized neutral protease: Immobilization enhanced the thermal stability and pH stability of neutral protease, and the optimal reaction pH changed from 7 to 9, indicating that immobilization conditions may change the conformation and active center of the enzyme.
3. Reuse performance of immobilized neutral protease: Immobilized neutral protease showed good stability in 10 consecutive reuses, although the initial activity decreased, but later activation efficiency tended to stabilize, maintaining an activity level of about 75%.

In summary, the neutral protease, especially after treatment by immobilization technology, has a significant promotion effect on the activation of TGase, and shows good stability and activity maintenance in repeated use.

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