Enzymatic Hydrolysis Effect of Different Components of Bacillus licheniformis on Globinase

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Abstract: To replace the globin with blood globulin powder made from pig blood, to explore the enzymatic hydrolysis effect of different components of Bacillus licheniformis on globinase, and provide evidence for further development and utilization of pig blood. Orthogonal test was carried out by using different reaction temperature, substrate concentration, reaction time and pH value. The enzymatic hydrolysis conditions were optimized, and the optimum conditions were determined by enzymatic hydrolysis. The components of the strain were determined by one-way experiment. Through this experiment, it was found that the five stages after the separation and purification of the protease from Bacillus licheniformis passed through the ultrafiltration membrane, and the component of 50-100 nm has the best enzymatic hydrolysis effect with globin. The 50-100 nm segmentation of Bacillus licheniformis by ultrafiltration separation has the best globin enzymatic hydrolysis effect. It can be screened for deep processing of pig blood, which has great application prospects for pig blood processing and environmental protection in China.

1. Introduction

Bacillus licheniformis showed that the cells showed a rod-like morphology under optical microscope and had enlarged cysts. In the culture dish, the dispersion of wrinkled and flattened bacterial colony with white surface was observed[1], which is one of the strains that has application potential in bacilli. In recent years, there have been more and more reports on the application of Bacillus licheniformis in various fields at home and abroad, and good research results have been achieved in industries such as medicine, feed processing, pesticides and aquatic products [2]. Especially in the field of feed processing research, the enzyme produced by Bacillus licheniformis has the characteristics of acid and alkali resistance, high temperature resistance and strong vitality [3], can produce different kinds of enzymes, and has the characteristics of a little loss in the process of production and processing of enzyme with high efficiency.

Different cell functions in the human body correspond to different types of active peptides [4]. Active peptides are the most important active substances in the human body. Because of the increase or decrease in the amount of secretion in the body, humans have a life cycle. And the timely supplement of the active peptide breaks the cycle of life, so that it can achieve the magical effect of prolonged life slowing down aging. In this study, pig blood globulin powder was used instead of globin, and different components isolated from Bacillus licheniformis were used to prepare the active polypeptide with ACE inhibition, which mainly have the functions that reduce blood pressure, anti-oxidation, anti-aging, improve immunity, lower cholesterol, prevents arteriosclerosis and iron supplementation [5]. Pig blood is rich in heme iron and has high utilization value. It can not only become the best choice for people to supplement iron element, but more importantly it is biomass iron,
which will not bring any toxic side effects caused by any other synthetic drugs.

2. Materials and methods

2.1 Materials

2.1.1 Strain
Bacillus licheniformis is provided by the Feed Science and Technology Research Institute of Anhui Agricultural University.

2.1.2 Medium
Fermentation medium: peptone 3.0g, corn syrup 0.9g, bean cake powder dipping juice 1:15, glucose 3.0g, pH=7.5, MPa=0.15 and sterilization for 25 min.

Seed culture medium: beef extract 5g, soybean peptone 10g, sodium chloride 5g, water 1L, pH=7.0, MPa=0.15 and sterilization for 25 min.

2.1.3 Main instruments and reagents
Globin (blood globulin powder made from pig blood), Weifang City Pharmaceutical Science and Technology Co., Ltd.; high-speed centrifuge, Beijing Times Béli Centrifugal Co., Ltd.; HYG-rotary constant temperature speed shake flask, Shanghai Yancheng Industrial Co., Ltd.; YX-280D pressure steam sterilizer, Hefei Huatai Medical Equipment Co., Ltd.; DHG series drying oven, Shanghai Sanfa Scientific Instrument Co., Ltd.

2.2 Experimental methods

2.2.1 Culture screening of Bacillus licheniformis
(1) Preparation of seed culture medium: First, prepare a fresh test tube. After sterilizing the inoculation loop, take Bacillus licheniformis from the preserved inclined surface and transfer to the fresh test tube slope, and incubate at 37 °C for 18 h. In the super-clean workbench, the inclined surface after 18 hours of culture was taken, and inoculated into a 250 ml triangular flask containing 50 ml of sterile seed culture medium with the first loop of sterile inoculating loop, and then set a rotation speed of 120r/min, 37 °C, and shaken culture for 24 hours in a rotary constant temperature speed shake flask.

(2) Shake flask culture: Take the seed liquid on the super-clean workbench, inoculate it in a 250 ml flask containing 50 ml of sterile seed culture medium with the second loop of sterile inoculating loop, and the inoculation amount is 8% (v/v). Set the rotation speed to 120r/min and 37 °C, and shake it in a rotary constant temperature speed shake flask for 24h.

(3) Take the cultured the Bacillus licheniformis by shake flask, inoculated into a 250 ml triangular flask containing 50 ml of sterile fermentation medium with the first loop of sterile inoculating loop, and then set the rotation speed to 120r/min, 37 °C, and shake it in a rotary constant temperature speed shake flask for 24h.

2.2.2 Isolation and purification of Bacillus licheniformis
(1) Elution. Using the saturated ammonium sulfate precipitation method of Chengsheng Liu [6]. Salting out: Taking the above fermentation liquor, after centrifugation at 5000r/min for 5min, add fresh ammonium sulfate solution with mass fraction of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%. After well shaken, let it stand at 6 °C overnight. Centrifuged it at 12000 r / min for 20 min at 4 °C, and the precipitate was dissolved in an appropriate amount of 50 mmol / L, pH = 7.0 Tris-HCl buffer, and then dialyzed against a buffer solution for 12 h.

(2) Cell disruption. The eluted target protein was placed in a high-speed grinding cell disruption machine at 500 r/min for 5 min.
2.2.3 Orthogonal optimization experiment of enzymatic hydrolysis of Bacillus licheniformis and globin

The experiment used enzymatic hydrolysis of the original culture solution, combined with the research of Haijin Liu et al [7] to select the reaction temperature, substrate concentration, reaction time, pH value, these four single factors to set three levels to conduct, L₉(3⁴) orthogonal experiment, the optimal combination of culture conditions was determined by using the degree of enzymatic hydrolysis as an indicator, and the verification test was carried out in Table 1.

Table 1 Orthogonal experiment factors and levels

| Level | A (Temperature(℃)) | B (Substrate concentration) | C (Reaction time (h)) | D (pH value) |
|-------|--------------------|-----------------------------|----------------------|-------------|
| 1     | 50                 | 6                           | 6                    | 8.0         |
| 2     | 53                 | 8                           | 8                    | 7.0         |
| 3     | 56                 | 10                          | 10                   | 7.5         |

2.2.4 Separation of different components to the globin enzymatic hydrolys solution experiment

(1) The super-filtration membrane is subjected to five segments of 500 to 800 nm, 200 to 500 nm, 100 to 200 nm, 50 to 100 nm, and 20 to 50 nm, and then the dry material level in the fraction is measured, and the final enzyme activity is calculated at the dry material level.

(2) The different components of the above five super-filtration segments are labeled as A, B, C, D, and E as test enzymes. Enzyme hydrolysis experiments were carried out in five groups of 500 ml beakers according to enzyme and protein concentration = 0.5%.

(3) Enzymatic hydrolysis: take 80g of blood globulin powder, add water to dissolve, add 0.4g of test enzyme, and react with electromagnetic stirrer in a constant temperature water bath for 10 hours at a certain temperature (design). Adjust pH with hydrochloric acid, heat up to 95 °C and sterilize for 1h. Then cool it to room temperature, adjust the pH to 9.0 with sodium hydroxide, and filter with a constant pressure to obtain a filtrate and a filter cake.

(4) The free amino acid, the degree of enzymatic hydrolysis, the dry material, and the peptide recovery ratio were measured.

2.2.4.1 Dry material measurement method

(1) The glass dish has constant weight, and the clean glass dish is placed in an oven, dried by heating at 100 °C for 4 h, taken out and placed in a dry oven for half an hour to be cooled, that is, A₁.

(2) After make the constant weight of the glass dish, accurately weigh a certain amount of the filtrate liquor in the glass dish. At this time, the total weight of the glass dish and the sample to be tested is A₂, put it into the oven, heat and dry at 100 °C, and take it out after 4 hours. After being placed in a dry oven for half an hour, it was weighed, that is, A₃.

Calculate \( DA = \frac{A₃ - A₁}{A₂ - A₁} \) (2-1)

Where, DA—dry material;
A₁—the quality of the constant weight glass dish;
A₂—the total weight of the sample and the glass dish;
A₃—the total weight of the dried sample and the glass dish;

2.2.4.2 Free amino acid calculation method (using potentiometric titration) [8]

(1) Instrument calibration.

(2) Take 12.5 ml of the filtrate sample and place it in a 250 ml volumetric flask. Add water to reach the scale, mix and take 10.0ml into a 250ml beaker, add 30ml of water, insert the glass electrode and mercury electrode, start the magnetic stirrer and titrate with NaOH standard titration solution, titrate with stirring, observe the indicator number, when it reach to acidity pH = 9.2, note the volume of the sodium hydroxide standard solution consumed. Add 10.0 ml of formaldehyde solution and mix.
titrate with NaOH standard solution to acidity pH=9.2, and record the volume V1 of the sodium hydroxide standard titration solution.

At the same time, taking 80 ml of water, first adjusted to pH=8.2 with NaOH solution, then add 10 ml of formaldehyde solution, and titrated to pH=9.2 with NaOH solution, and the volume V2 of the sodium hydroxide standard solution was recorded as a reagent blank test. Refer to Zeng Dong's related literature report [9] to calculate X.

\[
X = \frac{(V'2 - V1) \times C1 \times 0.014}{V'3 \times 12.5 / 250}
\]

Where, X- amino nitrogen content in the filtrate;
V1—the volume of the NaOH standard titration solution is consumed after the test solution is added with formaldehyde, ml
V2—blank test consumes NaOH standard drop solution volume, ml
V3—for test dosage, ml
C1—NaOH standard titration solution concentration, mol/l0.014—the standard titration solution with 1.00ml NaOH corresponds to the mass of nitrogen, g

2.2.4.3 Method for calculating the peptide recovery ratio [10]
(1) After the completion of the reaction, the total weight M' of the reaction liquor and the total weight m of the filtrate were weighed.

(2) The glass dish has constant weight, and the clean glass dish is placed in the oven. It was dried by heating at 100 °C for 4h, taken out and placed in a dry oven for half an hour to be cooled, that is, A1'.

(3) After constant weight of the glass dish, accurately weigh a certain amount of the reaction solution in the glass dish. At this moment, the total weight of the glass dish and the sample to be tested is A2', put it into the oven, and heat and dry at 100 °C. After 4 hours take it out and put it into the dry oven and cool it for half an hour and weigh it, that is, A3'.

Calculate DA' = \frac{A3' - A2'}{A2'}

Where DA' - dry material;
50 to 100 nm A2' - the total weight of the sample and the glass dish;
A3' - the total weight of the dried sample and the glass dish;

Peptide recovery ratio = \frac{filtrate volume \times filtrate dry material}{reaction liquid volume \times reaction liquid dry material} and \frac{m \times DA}{M \times DA''}

2.2.4.4 Enzymatic calculation method [11]
(1) After the completion of the reaction, take the total weight of M' of the reaction liquid and the total weight of filter cake m''.

(2) The glass dish is constant weight, and the clean glass dish is placed in the oven heat and dry at 100 °C for 4 hours, taken out and placed in a dry oven for half an hour to be cooled, that is, A1''.

(3) After constant weight of the glass dish, accurately weigh a certain amount of filter cake in the glass dish. At this time, the total weight of the glass dish and the sample to be tested is A2'', put into the oven, and heat and dry at 100 °C for 4h. After that, it was taken out and placed in a dry oven for half an hour and then weighed, that is, A3''.

Calculate DA'' = \frac{A3'' - A1''}{A2'' - A3''}

Where, DA''—dry material;
A1'' — the quality of the constant weight glass dish;
A2''—the total weight of the sample and the glass dish;
A3'' - the total weight of the dried sample and the glass dish;
Degree of enzymatic hydrolysis = \[ \frac{\text{filter cake volume} \times \text{filter cake dry material}}{\text{reaction liquid volume} \times \text{reaction liquid dry material}} \]

2.2.5 Data Statistics and Analysis
The data was sorted by Microsoft Excel software, the anova statement in SAS9.0 software was used for variance analysis, and the Duncan new complex range method was used for significant multiple comparison.

3. Results and analysis

3.1 Analysis of separation and purification of Bacillus licheniformis by saturated ammonium sulfate precipitation method
The supernatant liquid and the precipitate were separated and purified with 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% saturated ammonium sulfate, and the relative enzyme activity was determined as shown in Figure 1.

![Figure 1 Separation and purification of Bacillus licheniformis](image)

It can be seen from Figure 1 that when the ammonium sulfate saturation is from 10% to 30%, the relative enzyme activity of the supernatant liquid increases slowly with the increase of ammonium sulfate saturation, but the enzyme activity is as low as 16%. The precipitated relative enzyme activity decreases slowly with increasing ammonium sulfate saturation but the enzyme activity is as high as 90%. When the saturation of ammonium sulfate is from 30% to 70%, the relative enzyme activity of the supernatant liquid increases rapidly with the increase of ammonium sulfate saturation, and the relative enzyme activity of the precipitate decreases rapidly with the increase of ammonium sulfate saturation. There is an intersection between 50% to 60% of ammonium saturation; when the saturation of ammonium sulfate is from 70% to 90%, the relative enzyme activity of the supernatant liquid increases slowly with the increase of ammonium sulfate saturation and the enzyme activity reaches saturation. The relative enzyme activity of the precipitate decreases slowly with increasing ammonium sulfate saturation but the enzyme activity is very low.

Therefore, in this experiment, 30% saturation ammonium sulfate was used to remove precipitated protein, and then the supernatant liquid was supplemented with 80% saturation ammonium sulfate to precipitate the protein.
3.2 Orthogonal optimization experiment analysis of enzymatic hydrolysis of Bacillus licheniformis and globin.

Table 2 Orthogonal experimental results and analysis

| NO. | A   | B   | C   | D   | Enzymatic hydrolysis degree % |
|-----|-----|-----|-----|-----|--------------------------------|
| 1   | 1   | 1   | 1   | 1   | 6.78                           |
| 2   | 1   | 2   | 2   | 2   | 14.16                          |
| 3   | 1   | 3   | 3   | 3   | 21.01                          |
| 4   | 2   | 1   | 2   | 3   | 13.19                          |
| 5   | 2   | 2   | 3   | 1   | 16.39                          |
| 6   | 2   | 3   | 1   | 2   | 19.25                          |
| 7   | 3   | 1   | 3   | 2   | 11.70                          |
| 8   | 3   | 2   | 1   | 3   | 3.58                           |
| 9   | 3   | 3   | 2   | 1   | 3.62                           |
| K1  | 41.95 | 31.67 | 29.61 | 26.79 | 109.68                      |
| K2  | 48.83 | 34.13 | 30.96 | 45.12 |
| K3  | 18.9 | 43.88 | 49.11 | 37.77 |
| K⁰² | 1759.8025 | 1002.9889 | 876.7521 | 717.7041 |
| K¹² | 2384.3689 | 1164.8569 | 958.5216 | 2035.8144 |
| K₂² | 357.21 | 1925.4544 | 2411.7921 | 1426.5729 |
| S   | 163.8269 | 27.7998 | 79.055 | 56.7302 |
| R   | 29.93 | 12.21 | 19.5 | 18.33 |

The results of the analysis of the degree of enzymatic hydrolysis showed that $R_A > R_C > R_D > R_B$ can be seen in Table 2. It can be seen that the factor that has the greatest influence on the degree of enzymatic hydrolysis is the reaction temperature, followed by the reaction time and pH value, and finally the substrate concentration. The appropriate reaction temperature, reaction time, pH and substrate concentration can promote the enzymatic hydrolysis of globin (pig blood globulin powder) by Bacillus licheniformis. It can be seen from Table 2 that $A_2$ is the best condition in the factor A (reaction temperature), the best condition for the factor C (reaction time) is $C_3$, the factor D (pH) is the best with the $D_2$ condition, and the factor B (substrate concentration) is best in $B_3$, so the optimization condition is $A_2 C_3 D_2 B_3$. That is, the reaction temperature was 53 °C, the reaction time was 10h, the pH was 7.0, and the substrate concentration was 10%. In order to examine whether the influence of these factors is significant, the analysis of variance is shown in Table 3.

Table 3 Results of orthogonal variance analysis

| Variance source | Sum of deviation square $S$ | degree of freedom $f$ | mean square $s/f$ | $F$ | $P$ |
|-----------------|-----------------------------|-----------------------|------------------|-----|-----|
| A               | 163.8269                    | 2                     | 81.91345 | 5.8931 |
| C               | 79.055                      | 2                     | 39.5275  | 2.8437 |
| D               | 56.7302                     | 2                     | 28.3651  | 2.0407 |
| Error E'        | 27.7998                     | 2                     | 13.8999  |      |
| Sum T           | 327.2420                    | 8                     |              |     |

It can be seen from Table 3 that the significance of A is greater than that of C ($P<0.01$), and the significance of C is greater than that of D ($P<0.05$), Thus, the significance is: $A>C>D>B$.

3.3 Analysis of enzymatic hydrolysis of isolated different components and globin

The enzymatic hydrolysis experiment was carried out according to the preferred technique of the orthogonal experiment, and then the obtained substance was subjected to enzymatic hydrolysis to carry out a one-way factor experiment of component screening. The enzymatic hydrolysis experiments were carried out by the formula 2-1, 2-2, 2-3, 2-4 to determine the dry material, free amino acid, peptide recovery ratio, and enzymatic hydrolysis as shown in Figure 2.
It can be seen from Figure 2 that under the conditions of the best one-way factor combination A₂, B₃, C₃, and D₂, the free amino acids, peptide recovery ratio, enzymatic hydrolysis, and dry material measured in the experimental groups A, B, C, and D always show an upward trend; and the free amino acid, peptide recovery ratio, and enzymatic hydrolysis degree measured in the experimental group D reach the maximum of 0.17%, 72.4%, and 21%; however, the free amino acid and the degree of enzymatic hydrolysis were measured to be decreased in experiment E, but the dry material is the largest of the five groups. As can be seen from the comparison of Figure 2, it is found that group D has the best effect on enzymatic hydrolysis of globin, which is the components separated by 50 to 100 nm of Bacillus licheniformis.

4. Conclusions and discussion

Through this experiment, five stages after the separation and purification of the protease by Bacillus licheniformis passed through the ultrafiltration membrane were found, and the best effect of the enzymatic hydrolysis of globin was the component of 50-100 nm. However, the types of specific enzymes at 50 to 100 nm have not been studied herein. Bacillus licheniformis is an aerobic bacterium. This study controls four factors, reaction temperature, substrate concentration, reaction time and pH value. The reaction temperature was very important for the enzymatic hydrolysis. The reaction temperature of this study was chosen to be 50 °C, 53 °C and 56 °C. The results showed that when the reaction temperature was 53 °C, and the effect on enzymatic hydrolysis was the most significant. If the reaction temperature is too low, it will adverse to conduct the enzymatic hydrolysis, resulting in low enzyme activity. Excessive temperature will cause denaturation of the active substance in the cell and severely inhibit the metabolic cycle of the microorganism. The factor-reaction time played a second important role in enzymatic hydrolysis. The experiment was carried out at 6h, 8h and 10h. The results showed that when the reaction time was 10h, the effect on enzymatic hydrolysis was the most significant. The factor-pH value plays a third important role in enzymatic hydrolysis. The experiment selected pH 8.0, 7.0, 7.5 for the test. The results showed that when the pH value of 7.0 the enzymatic hydrolysis was the most significant. Because if the value is too low, it will affect the permeability of the cell membrane, which will affect the absorption of the nutrient and the secretion of protein by the cells.

Bacillus licheniformis is capable of metabolizing a variety of substances and is widely used in industry and medicine. It is a microorganism with high application value. The globin enzymatic hydrolysis solution has good plant-inducing activity, and can combine Bacillus licheniformis in plant antibacterial with globin enzymatic hydrolysis solution in plant-induced resistance to achieve better
pest and disease resistance. In this study, blood globulin powder made from pig blood was used instead of globin, so it can be used as a deep processing of pig blood, such as blood tofu, from the perspective of industrial production. From the perspective of environmental protection, it can greatly improve the ecological protection around the slaughterhouse to make sustainable economic development and improve people's needs for functional foods.

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