Preparation of low molecular weight chitosan by pawpaw protease

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Abstract. Pawpaw protease was used to degrade chitosan, and then obtained low molecular weight chitosan and reducing sugar in this study. According to the orthogonal experiment on the degradation of chitosan by pawpaw protease, the effects of temperature, pH value, reaction time and enzyme dosage on the decomposition efficiency of chitosan were investigated with the concentration of reducing sugar in the degradation solution as the index. The experimental results show that the optimal conditions for the degradation reaction are: the pH value of 4.1 and the reaction temperature of 42 °C, the reaction time of 1.4 h and the amount of enzyme of 30 U/mg, the concentration of reducing sugar in the degradation solution of 1.57 g/L and the yield of 15.7%.

1. Introduction

Compared with high-molecular chitosan, low-molecular-weight chitosan tastes with a refreshing sweetness that solubility, hygroscopicity and sweetness increases with the decrease of polymerization degree. It is an excellent food additive that possesses ability to improve the structure of food and increase the water retention and water activity of food [1]. On account of its physiological activities such as lowering cholesterol, enhancing human immunity, and resisting tumors and ulcers, low-molecular-weight chitosan is also used in the production of health foods. At present, Japan has developed a variety of health products made of low molecular weight chitosan, such as Carnegie, which has a blood lipid lowering function, and its main components are low molecular weight chitosan, organic iron and safflower oil [2]. Low molecular weight chitosan is also used in food preservatives, since it has antibacterial and antibacterial effects and is a novel of “green” food preservative, which has been widely promoted and applied.

The utilization of low molecular weight chitosan to inhibit tumors to prepare anticancer agents is one of the most important applications of low molecular weight chitosan in biomedicine, especially low molecular weight chitosan with a degree of polymerization of 6-8[3], which can be activated Lymphocytes in the human body thereby effectively inhibit the proliferation and the spread of cancer cells. Therefore, it has an extensive foreground in the development of anticancer drugs. Nanoparticles made of chitosan have the functions of targeting, alleviating and increasing drug absorption, thus have become a new hot spot in scientific research and development[4]. Lymphocytes in the human body are very sensitive to the pH change of the internal environment that need a weak alkaline environment. When the pH drops, its activity also decreases, which destroys the immune function of the human body. Oligochitosan can make the pH of body fluids tend to be weakly alkaline so that improves body immunity and increase the activity of lymphocytes to destroy cancer cells[5].
In this article, pawpaw protease was used to degrade chitosan to obtain low molecular weight and reducing sugar. The factors affecting the degradation of chitosan by pawpaw protease were determined according to orthogonal experiments.

2. Experimental

2.1. Orthogonal experiment of $L_{25}(3^5)$

Since the price of pawpaw protease is expensive, the orthogonal experiment of three factors and five levels is firstly carried out by using time, temperature and pH value, and the enzyme addition amount is 30 U/mg, thereby obtaining the best combination of these three factors.

2.2. Verification test

Experiments were carried out through the optimal reaction conditions obtained above verified the results of the experiments.

2.3. Measurement of Molecular Weight

The liquid exhibits viscosity (fluid between molecules) when flowing. Taking the viscosity of the solvent as $\eta_0$ and the viscosity of the polymer solution as $\eta$, the ratio of the viscosity $\eta$ of the solution to the viscosity of the solvent $\eta_0$ is called the relative viscosity, expressed as $\eta_r$. See the formula (1).

$$\eta_r = \frac{\eta}{\eta_0}$$  \hspace{1cm} (1)

When the viscosity of the solution is greater than the viscosity of the solvent, the increased fractional value is called the specific viscosity, expressed as $\eta_{sp}$. See formula (2).

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = \eta_r - 1$$  \hspace{1cm} (2)

So it can be known that $\eta_{sp}$ increases as the concentration of the polymer increases. The ratio of the specific viscosity to the concentration is referred to as the reduced viscosity, which indicates the increase in the viscosity of the solution caused by the unit concentration and is generally expressed as $\eta_c$. See formula (3).

$$\eta_c = \frac{\eta_{sp}}{C} = \frac{(\eta_r - 1)}{C} = \frac{(\eta - \eta_0)}{C \eta_0}$$  \hspace{1cm} (3)

The reduced viscosity when infinitely diluting the polymer solution is called intrinsic viscosity and is expressed as $[\eta]$.

The intrinsic viscosity is not directly related to the concentration of the solution, which represents the contribution of a single polymer to the viscosity of the entire solution.

In order to obtain the $[\eta]$ value, the reduced viscosity of several solution concentrations was determined in the experiment. According to the formula (4), $\eta_{sp}/C$ was plotted against $C$, extrapolated to $C=0$, and the obtained intercept was $[\eta]$, according to Equation (5), plotted against $\ln \eta_r/C$, extrapolated to $C=0$, and the resulting intercept is $[\eta]$.

$$\eta_{sp}/C = [\eta] + K[\eta]^2C$$  \hspace{1cm} (4)

$$\ln \eta_r/C = [\eta] - \beta[\eta]^2C$$  \hspace{1cm} (5)

Knowing the value of $[\eta]$, the average molecular weight can be calculated from the Mark-Houwink empirical equation $[\eta] = KM^a$. After the molecular weight ranges of the solvent system, temperature, high polymer and high polymer are determined, $K$ and $a$ in the above formula are constant.

$$\eta_{sp} = \frac{(t-t_0)}{t_0}$$

In the Formula:

- $t$ -- The time required for the solution to flow through the two lines of the viscometer.
- $t_0$ -- The time required for the solvent to flow through the two lines of the viscometer.
Therefore, $\eta_{sp}$ can be calculated as long as the $t$ and $t_0$ values are measured in the experiment, and then according to the above formula or , plot $\eta_{sp}/C$ versus $C$ or $\eta_r/C$ versus $C$ to obtain a straight line and extrapolate to $C=0$, and the resulting intercept is $[\eta]$.  

The Ubbelohde viscometer was vertically fixed in a constant temperature water bath, and the specified temperature was maintained at $30 \pm 0.02^\circ C$. 10 mL of the filtered chitosan solution was added to the Ubbelohde viscometer, and after the constant temperature of 10 min, when the solution temperature was balanced with the bath temperature, the outflow time of the solution was measured and the average value $t_1$ was taken for multiple measurements.

Then, using a pipette to add 5 mL of the solvent to the viscometer to dilute the solution concentration, the Ubbelohde viscometer was shaken to make the solution concentration uniform, and the measurement was performed again to obtain $t_2$.

The $[\eta]$ value was substituted into the $[\eta]=KM^a$ formula, and then the viscosity average molecular weight of chitosan was calculated.

3.  Result and Discussion

3.1. Orthogonal experimental data analysis and result

According to the experiment shown in Table 1, 25 experiments were carried out and the following experimental results were obtained. See Table 1 and Table 2 for details.

| Experimental factor | pH | Temperature (°C) | Time (h) | Experimental result |
|---------------------|----|------------------|----------|---------------------|
| 1                   | 3.8| 36               | 0.8      | 1.05                |
| 2                   | 3.8| 42               | 1.1      | 1.26                |
| 3                   | 3.8| 48               | 1.4      | 1.27                |
| 4                   | 3.8| 54               | 1.7      | 1.06                |
| 5                   | 3.8| 60               | 2        | 1.10                |
| 6                   | 4.1| 36               | 1.1      | 1.45                |
| 7                   | 4.1| 42               | 1.4      | 1.56                |
| 8                   | 4.1| 48               | 1.7      | 1.52                |
| 9                   | 4.1| 54               | 2        | 1.30                |
| 10                  | 4.1| 60               | 0.8      | 1.24                |
| 11                  | 4.4| 36               | 1.4      | 1.53                |
| 12                  | 4.4| 42               | 1.7      | 1.53                |
| 13                  | 4.4| 48               | 2        | 1.46                |
| 14                  | 4.4| 54               | 0.8      | 1.29                |
| 15                  | 4.4| 60               | 1.1      | 1.21                |
| 16                  | 4.7| 36               | 1.7      | 1.37                |
| 17                  | 4.7| 42               | 2        | 1.57                |
| 18                  | 4.7| 48               | 0.8      | 1.35                |
| 19                  | 4.7| 54               | 1.1      | 1.21                |
| 20                  | 4.7| 60               | 1.4      | 1.21                |
| 21                  | 5.0| 36               | 2        | 1.28                |
| 22                  | 5.0| 42               | 0.8      | 1.15                |
| 23                  | 5.0| 48               | 1.1      | 1.15                |
| 24                  | 5.0| 54               | 1.4      | 1.18                |
| 25                  | 5.0| 60               | 1.7      | 1.16                |
As shown in Table 2, the extreme values of pH, temperature and time of the factors become smaller in turn. Therefore, the primary and secondary relationship of the factors affecting the conversion rate was pH > temperature > time, so the pH value had the greatest influence on the experimental results, and the temperature was second, and the time was minimal. According to the degree of influence, the order of the factors from large to small and the average value of each factor were selected. The optimal values of each factor were 4.1 of pH, 42 °C of temperature, and 1.4 h of time.
3.2. Verification of experimental data analysis and result
The experiments were carried out under the conditions of 4.1 of pH, 42 ° of temperature, 1.4 h of time and 30 U/mg of enzyme dosage. The experimental data was 1.57 mg/mL, and the test results were found to be in accordance with the orthogonal experimental results.

3.3. Analysis and results of determination of molecular weight
See Table 3 and Table 4.

| Table 3. Determination and calculation of molecular weight before degradation. |
|---------------------------------------------------|------------------|------------------|
| Project                                           | Dissolvant NaAc-HAc | Chitosan solution |
| Apparent Viscosity/mPa-s                          | 1.03             |                  |
| First time through capillary time/s               | 58               | 59.6             |
| Second time through capillary time/s              | 57.8             | 59.7             |
| Third time through capillary time/s               | 57.9             | 59.6             |
| Average value/s                                   | 57.9             | 59.6             |
| Concentration/g·cm⁻³                              | 0                | 0.010            |
| Relative viscosity η_r                             |                  |                  |
| Increased specific viscosity η_sp                 | 1.029            | 0.029            |

Using a one-point empirical formula \([η]=3[η_sp]+3\ln η_r] / 4C\), \([η]=31.47\ cm^3/g\). According to the Mark-Houwink empirical formula \([η]=K\ M^α\), where \(K = 1.424 \times 10^{-3} \ cm^3/g\), \(α = 0.96\), the molecular weight \(M\) can be calculated to be 33 523.

| Table 4. Determination and calculation of molecular weight after degradation. |
|---------------------------------------------------|------------------|------------------|
| Project                                           | Dissolvant NaAc-HAc | Low molecular weight chitosan solution |
| Apparent Viscosity/mPa-s                          | 2.71             |                  |
| First time through capillary time/s               | 52.13            | 56.33            |
| Second pass through capillary time/s              | 52.03            | 56.22            |
| Third pass through capillary time/s               | 52.15            | 56.38            |
| Average value/s                                   | 52.1             | 56.31            |
| Concentration/g·cm⁻³                              | 0                | 0.0025           |
| Relative viscosity η_r                             |                  | 1.081            |
| Increased specific viscosity η_sp                 |                  | 0.081            |

Using a one-point empirical formula \([η]=3[η_sp]+3\ln η_r] / 4C\), \([η]=2.87 \ cm^3/g\). According to the Mark-Houwink empirical formula, the molecular weight can be calculated to be 2766.34.

4. Conclusion
In these experiments, the absorbance of degradation solution was determined by spectrophotometry, and then the concentration of reducing sugar was obtained. The concentration of reducing sugar was used as the basis for judging. The factors affecting the degradation of chitosan by pawpaw protease were discussed. The experimental results showed
that the optimum conditions for the degradation of chitosan by pawpaw protease were: Reaction temperature was 42 °C, 4.1 of pH, 1.4 of reaction time, and 30 U/mg of pawpaw protease. Under the reaction conditions, the molecular weight of the low molecular weight chitosan produced by the degradation was 2766.34, and the degradation yield was 15.7%. The degradation efficiency and economic benefit of chitosan were the best.

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