Supplementary Material

1. Optimization Tests for Cellulase Pretreatment

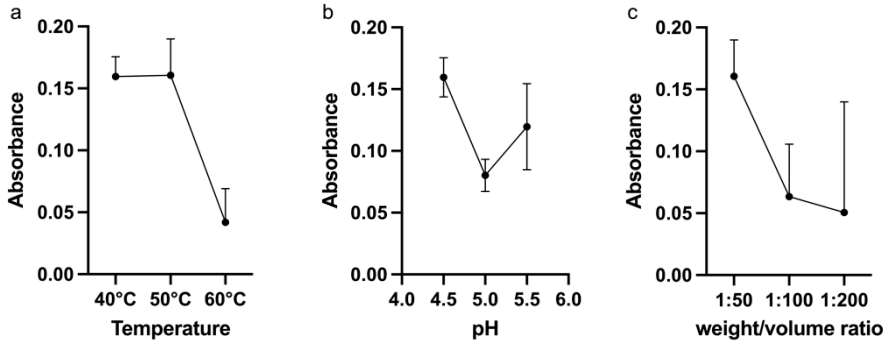
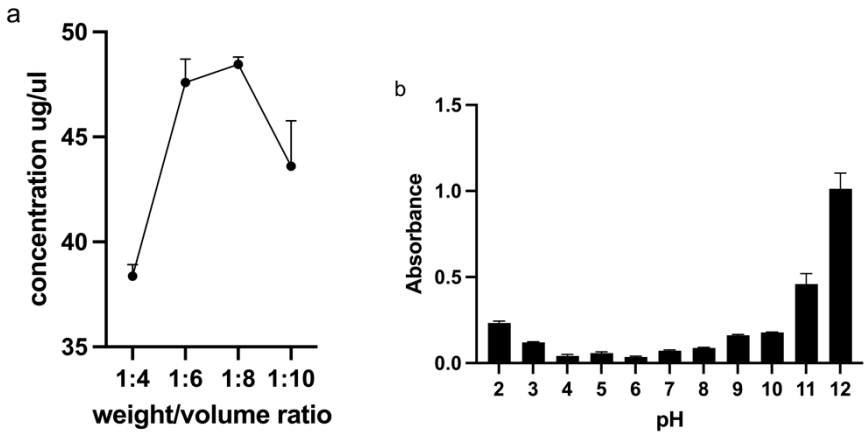


Figure S1. The effect of (a) temperature, (b) pH; (c) enzyme: BSG ratio on the protein extraction. Values represent the mean ± SD (n = 3). The absorbance of the sample is proportional to the concentration of protein in the sample, so the absorbance versus variables is used to represent the trends in the extracted protein content at different variables.

The cellulase used in this experiment is fermented and purified from the *Aspergillus Niger* strain. It refers to a collection of enzymes that can hydrolyze cellulose into glucose, mainly including endoglucanase, cellobiohydrolase, β -glucosidase. They act together to hydrolyze cellulose into glucose under the correct conditions. The effective temperature range is 40-60°C, the optimum pH range is 4.5-5.5, and the amount of enzyme added is 20-40U/g. Figure S1 shows the results of several sets of optimization experiments, which indicated that the optimal hydrolysis conditions of cellulase are a reaction temperature of 50°C, pH of 4.5, and enzyme ratio (enzyme/BSG aqueous solution) of 1:50.

2. Optimization Tests for Alkaline Extraction and Acidic Precipitation



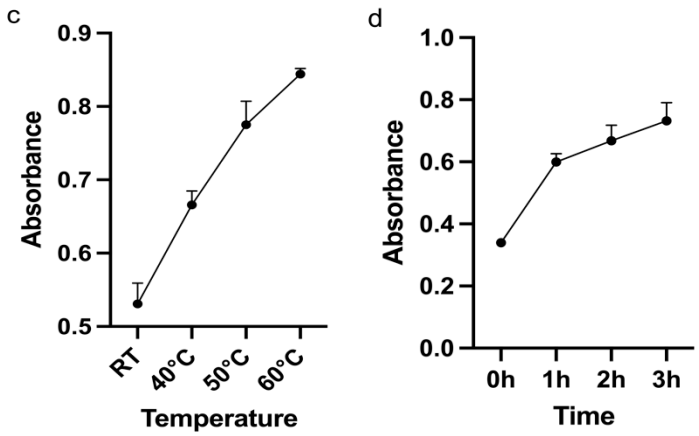


Figure S2. The effect of (a) weight/ volume ratio, (b) pH, (c) temperature; (d) reaction time on extraction of alkaline soluble protein. Values represent the mean ± SD (n = 3). The absorbance of the sample is proportional to the concentration of protein in the sample, so the absorbance versus variables is used to represent the trends in the extracted protein content at different variables.

The effect of the BSG: dH2O ratio on the alkaline extraction of protein from BSG was investigated over the range 1:4 to 1:10, with 1.00 M NaOH/ 1.00 M HCl for 1h at RT. The protein extraction rate increased significantly from 1:4 to 1:6, peaking at 1:8, but the growth was moderate, and as the ratio rose to 1:10, the protein extraction rate dropped considerably, so from the protein extraction efficiency and conserving water resources points of view, a weight/ volume ratio of 1:6 was chosen for the further extraction experiments.

The pH required for BSG protein isoelectric precipitation was explored. Precipitation occurs in the pH range of 6.0–3.0, according to the research. It is also worth noting that the BSG proteins are most soluble at pH 12. For recovery of the alkaline-extracted proteins from BSG, pH 3.5~4 was used as the precipitation pH and pH 11~12 was chosen as the solubilization pH in the subsequent tests. A previous study has found that pale BSG proteins have isoelectric precipitation values of pH 4.0 [1], which are equivalent to our findings.

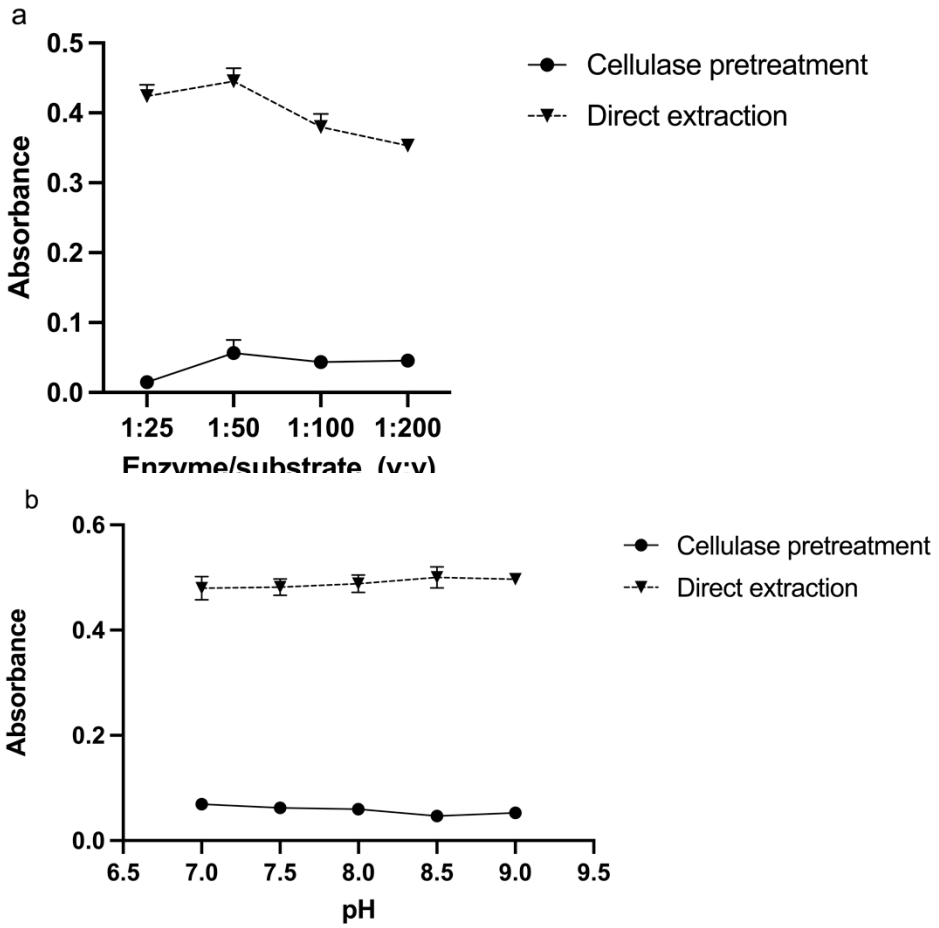
The impact of temperature on alkaline protein extraction from BSG was also tested over the temperature range of RT–60 °C. As the temperature increased, the amount of protein extracted from BSG with 1.00 M NaOH steadily increased. Temperatures above 60 °C were not studied because of the risk of protein denaturation. Earlier studies [1, 2] have demonstrated that raising the temperature improves the yield of alkaline extractable protein from BSG.

The reaction time was the last variable that was looked at in this study. As the reaction time increased, the protein yield increased, with the increase from 0h to 1h being the most significant, and the increase after 1h gradually becoming flat; 1h was chosen as the optimal reaction time for subsequent protein extraction to save time and environmental resources.

In the present work, the final conditions for extracting proteins from BSG were extraction with a 1:6 BSG: distilled water suspension, pH 12.0 adaptation, and reaction for 60 min at 60°C.

3. Optimization Experiment of Enzymatic Hydrolysis of Protein

3.1. Optimization Experiment of Alcalase Hydrolysis of Protein from Groups A and B


a
Absorbance
0.5
0.4
0.3
0.2
0.1
0.0
1:25
1:50
1:100
1:200
Cellulase pretreatment
Direct extraction
b
Absorbance
0.6
0.4
0.2
0.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
Cellulase pretreatment
Direct extraction

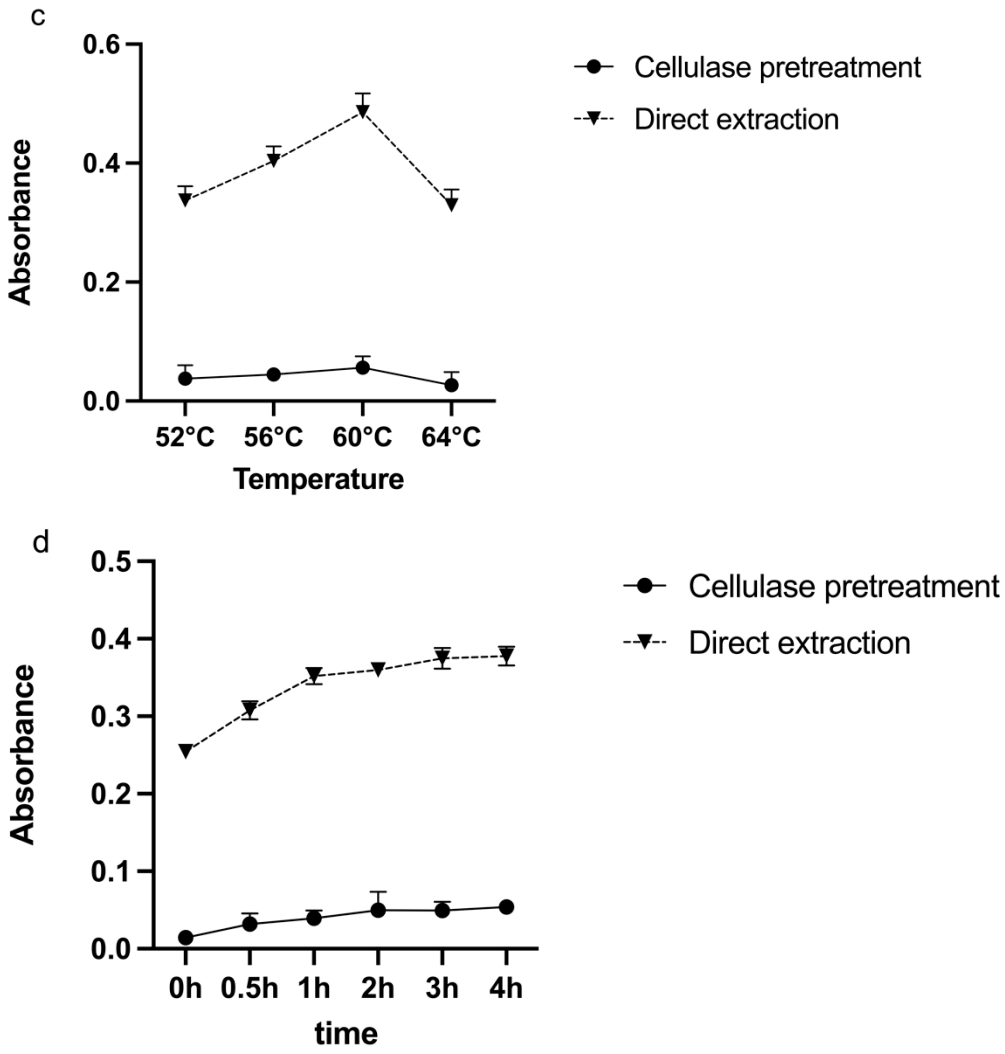


Figure S3. The effect of (a) Alcalase/ protein, (b) pH, (c) temperature; (d) reaction time on the degree of hydrolysis of alkaline soluble protein. Values represent the mean ± SD (n = 3). The absorbance of the sample is proportional to the DH of protein in the sample, so the absorbance versus variables is used to represent the trends of protein DH content at different variables.

In general, increasing the enzyme/substrate ratio improves the efficiency of protein hydrolysis and increases the hydrolysate yield. When the enzyme-to-substrate ratio reaches a certain point, the enzyme molecule is saturated with the substrate. Increasing the amount of enzyme does not seem to help much with increasing the degree of protein hydrolysis at this time. Furthermore, when the amount of enzyme is high, a large amount of hydrolysate is created at the start of the reaction, which might result in feedback inhibition of the enzyme, which is not good for the enzyme's reaction. As shown in Figure S3(a), whether or not BSG is pre-treated with cellulase, the DH reaches its highest value when the volume ratio of Alcalase to protein solutions is 1:50; therefore, this ratio is used for following protein hydrolyzation.

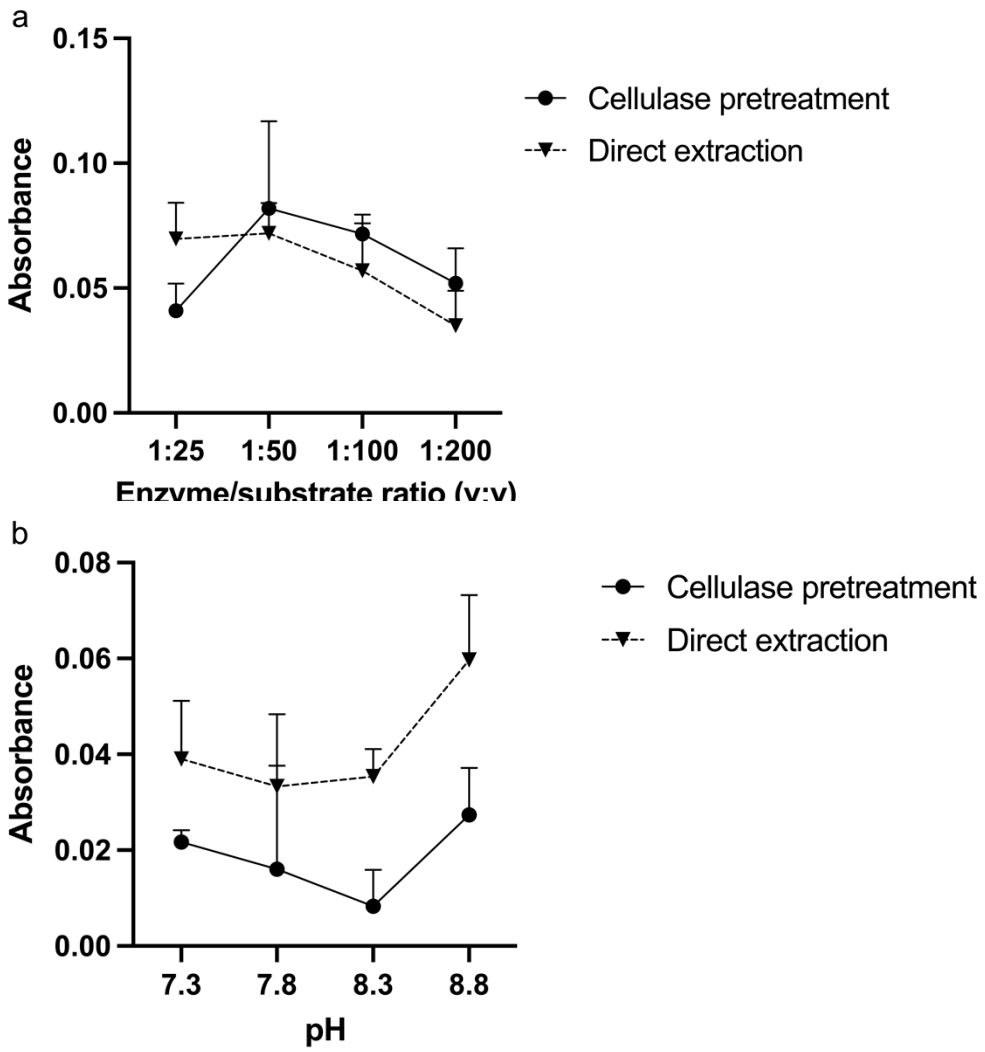
Each enzyme can only exhibit its activity within a specific pH range. The concentrations of hydrogen ion and hydroxide ion in the system affect the dissociation of the group and it can also influence the active center and enzyme conformation. Changes in pH in the medium influence the stability of the enzyme's molecular structure, and therefore influence the enzyme's activity. A suitable pH is not only beneficial for the enzyme's catalytic action but also protects the enzyme molecule and improves its stability. An overly high pH value, on the other hand, will be damaging to the enzyme and lead it to inactivate. As shown in Figure S3, the degree of hydrolysis of the directly extracted protein under Alcalase hydrolysis is much higher than that of the protein obtained after pretreatment

with cellulase. The directly extracted protein reaches its max at pH 8.5, as can be observed in Figure S3(b), and the change in pH has no significant effect on the protein obtained after cellulase pretreatment.

Any enzyme has its optimal reaction temperature. Low temperature inactivates the activity of the enzyme. As the temperature rises, the active group gradually increases. When the temperature exceeds its optimal value, the structure of the enzyme changes; enzyme activity is decreased. At the same time, temperature also affects the solubility of protease; thus, a too high or too low temperature is not suitable for hydrolysis. As observed in Figure S3(c), the trend in the degree of hydrolysis of the two groups of proteins is roughly the same. In the range of 52°C~60°C, as the temperature rises, the degree of hydrolysis increases significantly, but when the temperature reaches 64°C, the degree of hydrolysis decreases instead. Therefore, it is estimated that the optimal temperature value for the addition of enzymes for the two groups of proteins should be between 56°C and 64°C. It can be observed from the figure that the degree of protein hydrolysis is highest when the temperature is 60°C. Therefore, this temperature is selected as the most suitable temperature for Alcalase to hydrolyze these two groups of proteins.

Prolonging the reaction time can enhance protein yield, but it can also induce excessive hydrolysis of the protein, diminishing emulsification and foaming capabilities and producing a large number of bitter peptides. Furthermore, extending the reaction time means extending the production cycle; therefore, selecting an acceptable reaction time is critical to actual production. As observed in Figure S3 (d), the degree of protein hydrolysis steadily increases with time during the first hour of the reaction, with the increasing trend of the degree of protein hydrolysis becoming moderate between 1 and 3 h. The protein hydrolysis rate is extremely fast within the first hour. As the protein is hydrolyzed, a certain amount of protein, peptide, and amino acid accumulates in the solution and the enzyme is more likely to bind to the peptide in the liquid phase, thus reducing the amount of enzyme that acts on the protein. This is responsible for the delay of the rate of increase in the degree of hydrolysis. Protein hydrolysis is a reversible reaction and too much substrate will hinder the hydrolysis reaction. A long-term high-temperature environment, on the other hand, will inactivate a large number of enzymes. Furthermore, when considering the actual industrial production process, the benefits of increasing the degree of protein hydrolysis do not favorably compare with the cost increase, due to the prolonged reaction time. As a result, a reaction time of one hour is more reasonable in the research.

3.2. Optimization Experiment of α-chymotrypsin Hydrolyzing Protein from Groups A and B


a
0.15
0.10
0.05
0.00
Absorbance
1:25
1:50
1:100
1:200
Enzyme/substrate ratio (v:v)
Cellulase pretreatment
Direct extraction
b
0.08
0.06
0.04
0.02
0.00
Absorbance
7.3
7.8
8.3
8.8
pH
Cellulase pretreatment
Direct extraction

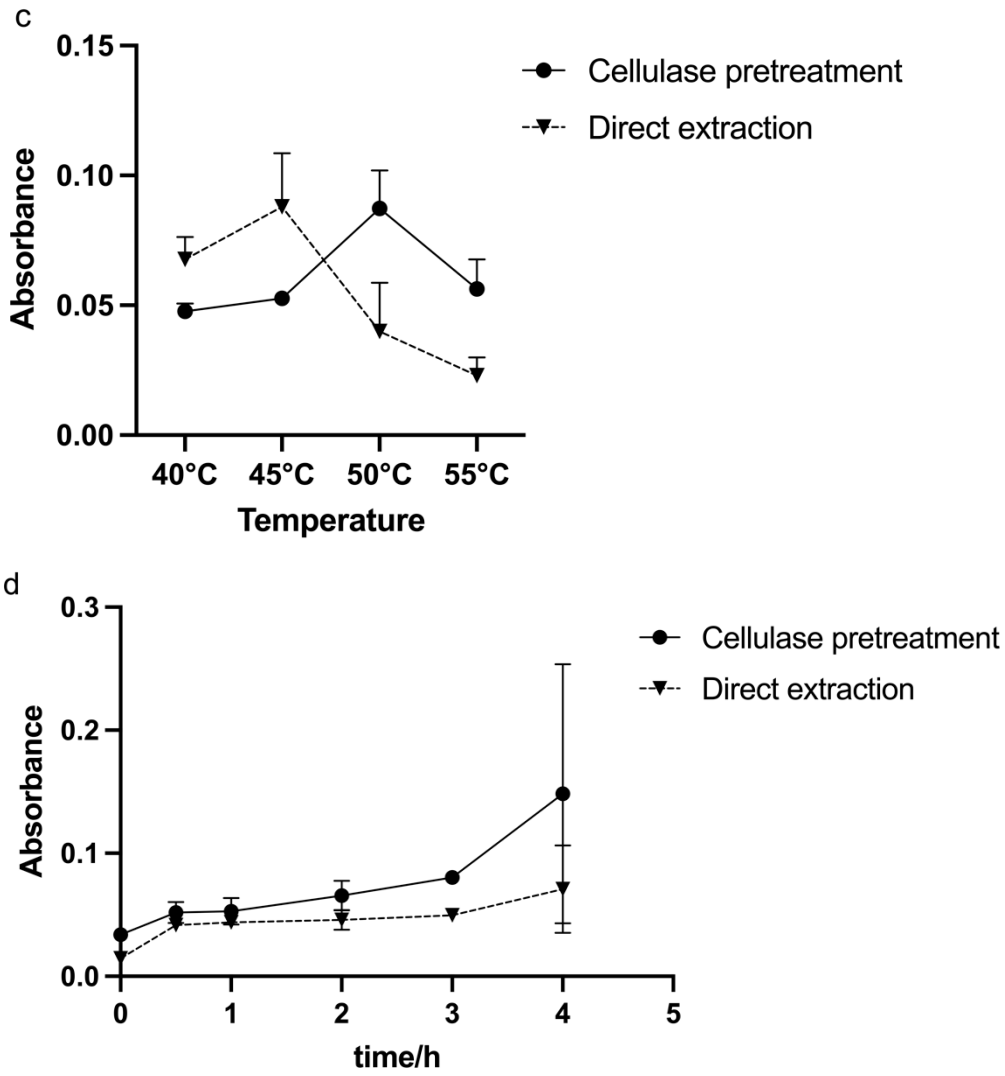


Figure S4. The effect of (a) α-chymotrypsin/ protein, (b) pH, (c) temperature; (d) reaction time on the degree of hydrolysis of alkaline soluble protein. Values represent the mean ± SD (n = 3). The absorbance of the sample is proportional to the DH of protein in the sample, so the absorbance versus variables is used to represent the trends of protein DH content at different variables.

α-Chymotrypsin is a protease with both proteolytic and esterolytic activity [3]. The appropriate temperature range for this protease is 40°C to 50°C, and the appropriate pH range is pH 7~8. A set of enzymatic hydrolysis optimization studies were designed based on α-chymotrypsin 's appropriate scope of activity.

As demonstrated in Figure S4 (a), the degree of hydrolysis of the two groups of proteins is greatest when the volume ratio of enzyme to protein solution is 1:50; therefore, this ratio is chosen for subsequent enzymatic hydrolysis.

As shown in Figure S4(b), when the pH climbed above 8.3, the degree of hydrolysis of the two groups of proteins increased significantly, peaking at pH 8.8; hence, pH 8.8 was chosen for subsequent protein hydrolysis.

As illustrated in Figure S4(c), the protein extracted directly by the alkaline approach had the highest degree of hydrolysis at 45°C, while the protein recovered after cellulase pretreatment had the highest degree of hydrolysis at 50°C. As a result, 45°C and 50°C were chosen as the enzymatic hydrolysis temperatures for the two protein groups, respectively.

From Figure S4(d), the degree of hydrolysis of the two groups of proteins increased significantly from 0h to 0.5h. Afterwards, the increasing trend of the degree of protein hydrolysis between 0.5h and 3h became stable and then accelerated after 3 h. Therefore,

in order to carry out a direct comparison with Alcalase, and from the perspective of practical consideration, 1h was selected as the reaction time of enzymatic hydrolysis.

In summary, experiments on the four factors of enzyme addition, pH, reaction temperature, and reaction time show that the optimal combination of factors for the hydrolysis with Alcalase for both groups of proteins are a 1:50 enzyme: protein ratio, pH 8.5, reaction temperature of 60°C, and reaction time of 1 h. Optimal α-chymotrypsin hydrolysis conditions include a reaction temperature of 45°C for directly extracted protein, 50°C for the cellulase-pretreated protein, and 1:50 enzyme to protein ratio, pH 8.8, and reaction time of 1 h for both. To our knowledge, no other studies on the enzymatic hydrolysis of cellulase-pretreated BSGP and α-chymotrypsin have been previously reported to be used to hydrolyze BSGP.

References

1. Celus, I; Brijs, K.; Delcour, J.A. Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. *J. Agric. Food Chem.* **2007**, *55*, 8703–8710.
2. Bals, B.; Balan, V.; Dale, B. Integrating alkaline extraction of proteins with enzymatic hydrolysis of cellulose from wet distiller's grains and solubles. *Bioresour. Technol.* **2009**, *100*, 5876–5883.
3. Flaschel, E.; Ebmeier, L. *Stabilization of α-Chymotrypsin by DMSO, in Progress in Biotechnology*; Elsevier: Amsterdam, The Netherlands, 1998; pp. 223–228.