Enzymatic Properties of Endopeptidase in Wheat Malt

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Abstract. Endopeptidase is the main enzyme in wheat malt. Besides, long chain proteins are broken down by endopeptidase into small protein molecules, or peptides, which can influence the beer quality. In this paper, enzymatic properties of endopeptidase in wheat malt were studied based on a more accurate method for the endopeptidase activity measurement. The activity of endopeptidase was determined by measuring the content of oligopeptides and polypeptides in the reaction system. It was found that the optimum temperature of wheat malt endopeptidase was 50 °C. It was shown that endopeptidase was sensitive to temperature and the low temperature is more suitable for endopeptidase. Further increase of temperature to values higher than 50 °C resulted in the decrease of enzyme activity. At 65 °C, the enzyme activity was decreased to 7.47 μ ± 0.42, then the enzyme can be deactivated at temperatures higher than 70 °C. The enzyme had optimal activity at pH 4.0. When the pH value was above 4, the enzyme activity cannot remain stable after incubation. The enzyme was inhibited by Ca²⁺, Pb²⁺, Zn²⁺, Cu²⁺, EDTA.

1. Introduction

Wheat is one of the major food crops in the world and China is the world's largest wheat producer. In 2018, China's wheat output reached 131.43 million tons. Wheat is the traditional material for beer production. In beer industry, beer brewed from wheat is regarded as a special type of beer [1]. In recent years, wheat beer is becoming more and more popular all over the world due to the increasing craft and home brewing and due to its' higher content of protein, amino acid and vitamin, and richer nutrition [2]. Moreover, wheat malt has been proved to it reduce cardiovascular disease and promote health [3]. However, the research about the changes of wheat malt during beer production is limited and further research is essential.

Wheat malt is rich in enzymes and nutrients. Baeza et al found that malting can increase the amount of health-promoting substances and releases the phenols [4, 5]. In addition, wheat malt also contains antisecretory factors [6, 7]. Compared with barley malt, wheat malt has higher protein content, lower tannin content and higher extract content [8]. The protein content and composition of wheat malt have important influences on the quality of finished beer. Therefore, wheat malt beer has higher amount of free amino acid and better degrading enzyme activity compared to barley malt, which further influence the flavor and durability of foam. Previous studies have found that soluble protein in malt plays an important role in the regulation of beer production. Beer foam stability is mainly
controlled by peptides and low-molecular proteins which were generally produced during malting process [9].

2. Materials and methods

2.1 Materials

The wheat malt was purchased from Supertime Malting Co., Ltd, China. The bovine serum albumin (BSA) were obtained from Solarbio, China. The biuret was purchased from Shanghai yuanye Bio-Technology Co., Ltd, China. All other chemicals used were commercially available.

2.2 Extraction of Crude Endopeptidase Enzyme

Wheat malt was grinded with EBC (European Brewery Convention) Mill. Then 40mL of 0.1M pH5.2 citric acid-hydrogen phosphate disodium buffer solution was slowly added to the beaker that containing 10g of wheat malt powder and stirred magnetically for 30min at 4°C. Then solution was fixed to 50 mL (V) by the same buffer and filtered at 4°C by the Qualitative filter paper. The obtained filtrate was used as crude enzyme solution.

2.3 Determination of Optimal Temperature

Optimal temperature of endopeptidase activity was determined by incubated in pH 4.5 acetic acid-sodium acetate buffer at different temperatures from 20 to 75°C for 4 hours. The enzyme activity assays were performed as described in section 2.3.

2.4 Determination of Thermal Stability

According to the results of 2.4, the reaction mixtures were incubated at different temperatures to determine the temperature-dependent stability. The thermal stability of the endopeptidase was investigated at four temperature points of 40, 45, 50, 60°C. The crude enzyme solution was incubated for 10 to 60 minutes at a temperature of 40 and 45°C and 50°C, respectively, and the gradient was 10 min. Then the crude enzyme solution was incubated at a temperature of 60 °C for 5, 10, 15, 25, 35, 45 min. Then the residual enzyme activity was measured. The enzyme activity assays were performed refer to section 2.3 and the highest enzyme activity at corresponding temperature point was defined as 100%.

2.5 Determination of Optimal pH

To estimate the optimal pH, crude enzyme was incubated in acetic acid-sodium acetate buffer at pH ranging from pH 3 to 6 at 50°C for 4 hours. After incubation, the endopeptidase activity was measured at 540 nm using BSA solution as substrate. The enzyme activity assays were performed according to section 2.3.

2.6 Determination of pH Stability

According to the results of 2.6, the reaction mixtures were incubated at different pH values (pH =4, 4.5, 5, 5.5) to determine the pH-dependent stability. The crude enzyme solution was incubated at pH 4, 4.5, 5, 5.5 for 10-60 min, respectively. The enzyme activity assays were performed according to section 2.3 and the highest enzyme activity at corresponding temperature point was defined as 100%.

2.7 Effect of Metal Ions and Inhibitors on Enzyme Activity

Metal ions and inhibitors (Ca2 +, Mg2 +, Zn2 +, Cu2 +, EDTA) were added to the enzyme solution to achieve the final concentration of metal ions of 5 mmol/L, and the enzyme solution was kept at 50°C for 10 min. Subsequently the enzyme activity of the endopeptidase was determined under the standard conditions (T=50°C, pH=4). Enzymatic activities were expressed as relative values (%) and the sample without any metal ions or inhibitors as control.
3. Results and discussion

3.1 Optimal Temperature

Figure 1 shows the changes of wheat malt endopeptidase activity as a function of temperature. As shown, the enzyme activity at 20°C was 0.61 u ± 0.21 and no significant difference of endopeptidase activity was detected between 20°C and 25°C. Increasing of temperature resulted in a significant increase of enzyme activity and reached the highest of 20.98 u ± 1.60 at 50°C. This is because higher temperatures cause molecules to move faster and increases the probability of collisions, which speeds up the reaction rate. Further increase of temperature to values higher than 50°C resulted in the decrease of enzyme activity. At 65°C, the enzyme activity was decreased to 7.47 u ± 0.42. It can also be seen that the enzyme can be deactivated at temperatures higher than 70°C.

3.2 Thermal Stability

Figure summarizes the thermal stability of the endopeptidase over time at 40°C, 45°C, 50°C and 60°C respectively. Apparently, wheat malt endopeptidase activity remained stable up to 60 min at mild temperatures of 40, 45 and 50°C. In contrast, at 60°C, the enzyme activity decreased rapidly with incubation time. The activity decreased to 78.15% at 25 min and to 67.17% at 45 min. The results indicated that the enzyme activity is stable between 40°C to 50°C. Due to the denaturation of enzyme protein by temperature accumulates with time increased. When the temperature was low, the enzyme protein itself was relatively stable, and the reaction time can be longer; on the contrary, the heat denaturation of enzyme increases with time increased, the action time cannot be too long at high temperature.

Figure 1. Effect of temperature on the activity of endopeptidase.

Figure 2. The thermal stability of the endopeptidase. A) 40°C, 45°C, 50°C B) 60°C
3.3 Optimal pH

pH is an important factor that will influence the activity of enzymes. In this study, the endopeptidase activity in wheat malt was studied from pH 3 to 6 and the results were shown in Figure 3. Since pH affects the dissociation state of substrate molecules, it affects enzyme in combination with substrate. At pH3.0, the enzyme activity was 9.66u±0.25. The increase of pH to 3.5 significantly increased the enzyme activity to 17.13u±0.98. A maximum value of 20.98u±0.60 at detected at pH4.0. However, further increase of pH led to the decrease of enzyme activity. At pH4.5, the enzyme activity was 13.20u±0.55%, and it decreased rapidly to 5.72u±0.27 when the pH was increased to 5.0. No further decrease was noticed between pH 5.5 and pH 6.0.

![Figure 3. Effect of pH on the activity of endopeptidase.](image)

3.4 pH Stability

Figure 4 indicated the stability of wheat malt endopeptidase at different pH values. At the lowest pH (4.0) studied in this research, the enzyme activity was very stable and no significant change was detected even at the highest incubation time of 60 min. On the contrary, a slight decrease of enzyme activity with increase of time was observed at pH 4.5 and about 71.64% of enzymatic activity was remained at 60 min. The stability of enzyme activity was further decreased with the increasing pH, the values decreased to 39.03% and 30.13% after incubation of 60 min at pH 5.0 and 5.5 respectively.

![Figure 4. The pH stability of the endopeptidase.](image)

3.5 Effects of Metal Ions and Inhibitor on Endopeptidase Activity

Metal ions can bond with the active groups in the active centers of small molecules or enzyme molecules, and this may cause the decrease of enzyme activity. The effect of metal ions and inhibitor on the endopeptidase activity is presented in Table 1. It can be observed that the addition of 5mM Ca2+, Pb2+, Zn2+, Cu2+, EDTA inhibited the enzyme activity significantly. Among all metal ions,
Ca\textsuperscript{2+} had the strongest effect on enzyme activity as only 57.71% can be remained. In contrast, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} only slightly decreased the enzyme activity to 86.74% and 72.76%.

| inhibitors | Relative activity (%) |
|------------|-----------------------|
| Cu\textsuperscript{2+} | 86.74±0.56b |
| EDTA | 63.08±0.18d |
| Pb\textsuperscript{2+} | 65.59±0.28d |
| Zn\textsuperscript{2+} | 72.76±0.09c |
| Ca\textsuperscript{2+} | 57.71±0.28e |
| Control | 100±0.46a |

4. Conclusions
The present study investigated the activity of wheat malt endopeptidase as a function of temperature, pH, time and metal ions. The optimum conditions for this enzyme are at pH4 and temperature 50\textdegree C. At this condition, the endopeptidase exhibited great stability up to a incubation time of 60 min. Meanwhile, the low Km value calculated from linear fit of Lineweaver Burk plot indicated that the enzyme and the substrate have great affinity. The addition of Ca\textsuperscript{2+}, Pb\textsuperscript{2+}, Zn\textsuperscript{2+}, Cu\textsuperscript{2+}, EDTA inhibited the enzyme activity significantly.

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