Properties of Amylase from Germinated Waxy Wheat Seed

Akiko YAMADA TANIGUCHI1,2, Shuhei KIKUCHI2 and Katsumi TAKANO2
1 Kamakura Women’s University, Ofuna, Kanagawa 247-8512
2 Department of Biosciences, Tokyo University of Agriculture, Setagaya, Tokyo 156-8502
E-Mail: akiko@kamakura-u.ac.jp

The amylases of germinated waxy wheat seed were purified by ammonium sulfate precipitation, with subsequent ion-exchange chromatography, chromatofocusing, and gel chromatography. Three different amylases were obtained in a pure state and are tentatively designated as amylases I, II and III, each yielding a single band when examined by electrophoresis. The specific activities of amylases I, II and III were respectively 5.1, 23- and 0.9-fold that of the crude enzyme extract. Amylases I, II and III had respective molecular weights of 56,000, 64,000 and 53,000. Amylase I showed the highest activity at pH 5.5 and 50°C, amylase II showed it at pH 6.0 and 50°C, and amylase III showed it at pH 6.5 and 50°C. Amylases I, II and III were stable from pH 4.5-7.0 and below 50°C. All activities of the enzymes were inhibited by mercury, PCMB, Ni2+, Cd2+ and Cu2+ which may indicate that an SH group was necessary for activating the three enzymes. Amylases I and II specifically hydrolyzed amylopectin, while amylase III did not act much on amylopectin, but did decompose pullulan. A qualitative thin-layer chromatographic analysis of the products of this digestion revealed enzyme specificity. The decomposition products revealed that amylase I was of β form, amylase II was of α form, and amylase III was a maltopentaose-forming enzyme.

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Key words: waxy wheat, amylase, properties, germinated seed

Materials and Methods

Test sample

Seeds of waxy wheat (a waxy gene-deficient mutant of an ordinary Triticum aestivum L line) grown at the Tohoku Agricultural Experiment Station were immersed in water at 20°C in the dark for 20 hours for germination and used for the subsequent experiment.

Crude enzyme preparation

A 0.1 M phosphate buffer solution (pH 6.0) of 20 times...
as much in weight was added to the germinating seeds whose cells had been disrupted with a Physcotron (Nichion Medical & Scientific Instruments) at 10,000 rpm. The treated seeds were stirred at 4°C for one hour to extract the enzyme. The resulting liquor was centrifuged (10,000 x g for 10 minutes), the supernatant being salted out with ammonium sulfate (80% saturated) and the precipitate being dissolved in a 20 mM phosphate buffer solution (pH 6.0). The resulting solution was dialyzed to prepare a crude enzyme solution.

**Amylase activity measurement**

To 0.5 mL of the crude enzyme solution, 0.5 mL of a 1% soluble starch solution (dissolved in the 0.1 M phosphate buffer solution of pH 6.0) was added. After reacting at 40°C for 10 minutes, the Somogyi-Nelson method was used to quantify the reducing sugar for determining the amylase activity. One unit of the enzyme is defined as its amount to form 1 μmol of the reducing sugar (maltose) in 1 minute.

**Protein quantification**

Protein was quantified by an improved version of the Lowry method.

**Chromatography**

- **DEAE-cellulose ion-exchange chromatography.**
  The crude enzyme solution was loaded into a DEAE-cellulose column (1.5 x 15 cm) that had been equilibrated with a 20 mM tris-HCl buffer solution (pH 8.0). After passing the buffer solution through the column, 200 mL each of a 20 mM tris-HCl buffer solution (pH 8.0) and a 0.7 M sodium chloride solution was used for gradient elution. Sequential 5-mL fractions of the eluate were measured for amylase activity and for protein by ultraviolet absorption at 280 nm.

- **Chromatofocusing.**
  A column (1.2 x 18 cm) was filled with a polybuffer exchanger (PBE, Pharmacia Corporation) buffered with a 25 mM imidazole-HCl solution (pH 6-8). The column was eluted with 100 mL of a polybuffer 96:97 (1:2)-HCl solution (pH 4-6). Sequential 3-mL fractions of the eluate were measured for amylase activity and for protein (280 nm).

- **Gel chromatography.**
  To a Sephadex G-100 gel column (1.5 x 85 cm) buffered with the 20 mM phosphate buffer solution (pH 6.0), the active fraction obtained in the preceding chromatofocusing step was added. The column was eluted with 300 mL of the buffer solution (pH 6.0). Sequential 3-mL fractions of the eluate were measured for amylase activity and protein (280 nm).

**Purity determination**

Disc electrophoresis was used to determine the purity, in which the gel for isolation (10% polyacrylamide, pH 8.3) was prepared according to the method of Nagai, and protein (5 μg) was added for migration at 2 mA per gel strand. Protein was stained with Coomassie brilliant blue.

**Molecular weight measurement**

Gel filtration and SDS-polyacrylamide gel electrophoresis were used for measuring the molecular weight. Gel filtration used an enzyme solution (0.1 mg as protein) and standard protein solutions added to a Sephadex G-100 gel column (1.5 x 100 cm) buffered with the 20 mM phosphate buffer solution (pH 6.0) containing 1 mM calcium chloride. The buffer solution was used to take sequential 2-mL fractions of the eluate at a rate of 12 mL/h. The positions of the fractions were compared with those of standard proteins to estimate the molecular weights. The standard proteins used (Boehringer Mannheim & Yamanouchi) were cytochrome c (molecular weight of 12,500), chymotrypsinogen A (25,000), hen egg albumin (45,000), and bovine serum albumin (68,000).

SDS-polyacrylamide gel electrophoresis was run after bonding the enzyme proteins to SDS and adding to the 10% polyacrylamide isolation gel for migration at a constant electric current of 8 mA/strand. The migration positions were compared with the molecular weights of standard proteins. The standard proteins used (Boehringer Mannheim & Yamanouchi) were α₂-macroglobulin (molecular weight of 170,000), phosphorylase b (97,400), glutamate dehydrogenase (54,000), lactate dehydrogenase (36,500), and a trypsin inhibitor (20,100). The proteins were stained with Coomassie brilliant blue.

**Isoelectric point measurement**

Rotophor electrophoresis apparatus from Bio-Rad Laboratories and pH 3-10 Bio-Lyte from Bio-Rad Laboratories were used. After migration (4°C) at 300V for four hours, 2-mL fractions of the solution were taken and measured for their amylase activity and pH value to determine the isoelectric points.

**Substrate specificity**

To 0.5 mL of an enzyme solution (10 units), 5 mL of a 2% solution of soluble starch, amylopectin, one of two kinds of amylose (molecular weights of 2,900 and...
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160,000), pullulan, α-cyclodextrin, or β-cyclodextrin was added for a reaction at 40˚C for 10 minutes. The amylase activity was measured according to above-mentioned method. Each substrate had been dissolved in a 1% (v/v) dimethylsulfoxide solution.

**Examination of the decomposition products**
To 0.5 mL of a 0.2% amylose suspension (Tokyo Kasei Industry Corporation), 0.1 mL of an enzyme solution (5 units) was added for a reaction at 40˚C for 10 hours. A heat treatment at 100˚C for 10 minutes was used to stop the reaction. The heated suspension was then concentrated to 0.1 mL under vacuum to prepare a sample solution. The sample solution (2 μL) was spotted on to a Kieselgel 60 F 254 thin-layer plate (Merck & Company) for parallel development at room temperature. After this development, the thin-layer plate was sprayed with a 5% (w/v) sulfuric acid-methanol solution and heated at 100˚C for 30 minutes to detect the spots. The standard substances used were glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.

**RESULTS AND DISCUSSION**

**Amylase purification**
Fig. 1 shows the relationship between amylase activity and the state of seed germination for nine days after sowing. As had been found with germinating seeds of Italian millet, Japanese millet, and Chinese millet, the buds from waxy wheat seeds grew rapidly in the fourth and fifth days after sowing, and the amylase activity reached a maximum of 14,000 units/10 g of seed on the fifth day, this being nearly 14 times higher than that of the non-germinating seeds. A crude enzyme solution prepared from seeds on the fifth day of germination was therefore subjected to DEAE-cellulose chromatography in this experiment. Fig. 2 shows the result. Amylase activity was found near sodium chloride concentrations of 0.17 M, 0.25 M and 0.3 M, the ratio of these activities being 2.2:1.6:1.

These three active fractions designated as fractions I, II and III were then purified by chromatofocusing. As shown in Fig. 3, fraction I had peak activity at around pH 7.1, fraction II at around pH 5.3, and fraction III at around pH 4.7. A further treatment by gel chromatography yielded a single peak in each fraction which was tested for purity by disc electrophoresis. Each of fractions I, II and III showed a single protein band, confirming that they were had been highly purified. The amylases in fractions I, II and III, which are designated as amylase I, amylase II and amylase III, were then examined for the properties described in the next section. Table 1 gives their specific activities and activity recovery ratios in the purification stages. The specific activities of amylases I, II and III were 30, 21, and 18 times as high as those of the extract solution, and the respective activity recovery ratios were 5.1%, 2.3% and 0.9%.

![Fig.1. Relationship between amylase activity and the state of seed germination](image)
Length of bud was expressed as average of length of bud.
- ● - Amylase activity, - ◆ - Length of bud

![Fig.2. Ion-exchange chromatography of the germinating waxy wheat amylase on DEAE-cellulose](image)
Crude enzyme, after (NH₄)₂SO₄ precipitation, was applied on a DEAE-cellulose column (1.5 × 15cm) previously equilibrated with 20mM Tris-HCl buffer (pH 8.0). The enzyme was eluted by the linear gradient with 20mM Tris-HCl buffer (pH 8.0) and 0.7M NaCl solution (400ml).
- ◆ - protein (absorbance at 280nm); - ● - Amylase activity
Properties of the purified enzymes

Effect of pH.

McIlvaine buffer solutions (pH 3.5-7.0) were used to find the optimum pH values (Fig. 4) and pH stability of the amylases. The optimum pH values were 5.5, 6.0 and 6.5 for amylases I, II and III, respectively. These optimum pH values were in the more acidic range than those for the β-amylases of barley and wheat malt (pH 5.2)\(^{10}\), and for the amylases of germinating seeds of Italian millet (pH 5.5)\(^{11}\) and Japanese millet (pH 6.0)\(^{2}\). Amylases I, II and III were all stable at 40°C for 30 minutes in a pH range of 4.5-7.0. This pH range for amylase stability is narrower than that for wheat amylase (pH 4.5-9.2)\(^{10}\) and for barley amylase (pH 4.5-8.0)\(^{10}\).

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Table 1. Purification process for the germinated waxy wheat amylase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract I</td>
<td>720</td>
<td>143.6</td>
<td>1.99</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Crude enzyme</td>
<td>316</td>
<td>80.45</td>
<td>2.55</td>
<td>1.3</td>
<td>56</td>
</tr>
<tr>
<td>DEAE-cellulose I</td>
<td>55.4</td>
<td>24.62</td>
<td>4.44</td>
<td>2.2</td>
<td>17</td>
</tr>
<tr>
<td>DEAE-cellulose II</td>
<td>27.2</td>
<td>18.83</td>
<td>9.05</td>
<td>4.5</td>
<td>13</td>
</tr>
<tr>
<td>DEAE-cellulose III</td>
<td>14.1</td>
<td>11.30</td>
<td>8.01</td>
<td>4.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Chromatofocusing I</td>
<td>0.711</td>
<td>11.13</td>
<td>15.7</td>
<td>7.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Chromatofocusing II</td>
<td>0.320</td>
<td>0.521</td>
<td>16.3</td>
<td>8.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Chromatofocusing III</td>
<td>0.212</td>
<td>0.347</td>
<td>16.5</td>
<td>8.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Gel filtration I</td>
<td>0.122</td>
<td>0.726</td>
<td>59.5</td>
<td>30</td>
<td>5.1</td>
</tr>
<tr>
<td>Gel filtration II</td>
<td>0.081</td>
<td>0.335</td>
<td>41.3</td>
<td>21</td>
<td>2.3</td>
</tr>
<tr>
<td>Gel filtration III</td>
<td>0.035</td>
<td>0.128</td>
<td>36.3</td>
<td>18</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\(^{1}\) 10g of waxy wheat seed was used.

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Fig. 3. Chromatofocusing or the germinating waxy wheat amylase

The each fraction (I, II and III) after DEAE-cellulose chromatography was applied on a polyexchanger gel column (PBE94, 1.5 × 85cm) previously equilibrated with 20mM imidazole-HCl buffer (I: pH8.0, II: pH7.0, III: pH6.0), and then the enzyme was eluted with the polybuffer96-97-HCl buffer.

- ◆ - protein (absorbance at 280nm).
- ○ - Amylase activity.
- − - pH.
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- Temperature effect.

The optimum temperature was 50°C for all of amylases I, II and III (Fig. 5), this being close to that for the amylase of germinating seeds of Japanese millet (40°C). The respective activities of amylases I and II at 60°C declined to about 80% and 85%, while the amylase III activity dropped to about 20%. This low activity at 55-60°C implies that amylase III, unlike amylases I and II, would be unstable at temperatures higher than 50°C.

Fig. 4. Effect of pH on the activity of waxy wheat amylase
Buffers used were 0.1M McIlvaine buffer (pH3.5-7.0).
The reaction was carried out for 10 min with pH alteration (at 40°C), and the activity was expressed as a percentage of the maximum activity. ●, Amylase activity.

Fig. 5. Effect of temperature on the activity of waxy wheat amylases
The reaction was carried out for 10 min with temperature alteration (I: pH5.5, II: pH6.0, III: pH6.5), and the activity was expressed as a percentage of the maximum activity. ●, Amylase activity.
Molecular weight.

SDS-polyacrylamide electrophoresis and gel filtration were used to measure the molecular weight. As shown in Table 2, amylases I, II and III were estimated to have molecular weights of 56,000, 64,000 and 53,000, respectively. Both methods gave the same values for molecular weight, showing that the amylases were all a single-strand polypeptide. The molecular weight of amylase I was the same as those of the β-amylases of germinating Italian millet seeds (56,000) and barley malt (56,000), while amylase III had a molecular weight close to that of the β-amylase of rice (53,000).

Table 2. Properties of the germinated waxy wheat amylases

<table>
<thead>
<tr>
<th>Properties</th>
<th>Amylase I</th>
<th>Amylase II</th>
<th>Amylase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>56,000</td>
<td>64,000</td>
<td>53,000</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>56,000</td>
<td>64,000</td>
<td>53,000</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>56,000</td>
<td>64,000</td>
<td>53,000</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>7.2</td>
<td>5.4</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Isoelectric point.

Isoelectric point electrophoresis was performed to make measurements (Table 2). The isolectric points were 7.2, 5.4 and 4.8 for amylases I, II and III, respectively. The isolectric point for amylase I was on the alkaline side of those for the β-amylases of barley malt (pl 6.0) and wheat (pl 6.9); the isolectric points for amylases II and III were on the acidic side. Amylases II and III had the same respective isolectric points as those for the β-amylases of Italian millet and Japanese millet.

Effects of various reagents.

Various reagents were added to the enzyme solutions (10 units) at a reagent concentration of 1 mM. After keeping the enzyme solutions at 40°C for 30 minutes, the residual enzyme activities were measured and are expressed relative to 100% for the enzyme activities without adding the reagent. Table 3 shows complete inhibition of amylases I, II and III by HgCl₂ and p-chloromercuribenzoic acid (PCMB), indicating the involvement of the SH group in the expression of the enzyme activities. Ni²⁺, Cd²⁺, and Cu²⁺ also considerably decreased the activities of the three enzymes to less than 38%. In addition, Pb²⁺ and Zn²⁺ respectively reduced the amylase III activity to 12% and 21%; the metals were found to vary in their effects on the activities of amylases I, II and III.

Decomposition products.

Thin-layer chromatography was used to examine the decomposition products of amylose by the enzymes. As shown in Fig. 6, only maltose was detected as a decomposition product of amylase I which was a β-amylase that decomposed starch into maltose units. In contrast, decomposition by amylase II yielded such oligosugars as maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, and maltose, confirming amylase II to be an α-amylase. Amylase III yielded only maltopentaose, revealing amylase III to be a maltopentaose-forming enzyme. Several microbial origins, including Bacillus licheniformis, of pentaose-forming enzymes have been reported. However, there have been few studies on the plant origins of the enzyme. There has been a study on the purification of three amylases from yam (Dioscorea dumetorum), one of which was reportedly a maltopentaose-forming enzyme. Plant amylases have been surmised to have specific mechanisms of action as just described and to play certain roles.

Table 3. Effect of various reagents on the activity of the germinated waxy wheat amylases

<table>
<thead>
<tr>
<th>Reagent (1mM)</th>
<th>Amylase I</th>
<th>Amylase II</th>
<th>Amylase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>96</td>
<td>94</td>
<td>40</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>88</td>
<td>95</td>
<td>51</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>86</td>
<td>93</td>
<td>12</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>83</td>
<td>87</td>
<td>62</td>
</tr>
<tr>
<td>KCl</td>
<td>82</td>
<td>82</td>
<td>50</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>50</td>
<td>61</td>
<td>21</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>7</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>4</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH₂COOH</td>
<td>96</td>
<td>71</td>
<td>26</td>
</tr>
<tr>
<td>EDTA²</td>
<td>98</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>PCMB³</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ The enzyme was preincubated with 1mM of various reagents at 40°C for 30 min, and the remaining activity was determined and is expressed as a percentage of the activity without addition.

² EDTA: ethylenediaminetetraacetic acid

³ PCMB: p-chloromercuribenzoic acid
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Table 4 shows a comparison of the actions of the enzymes on various substrates. Amylase I (α-β-amylase) and II (α-amylase) both exhibited 2-2.4 times higher decomposition activities than soluble starch toward amylopectin; the activity of amylase III toward amylopectin was about one fourth. Amylases I and III showed high decomposition activities toward a macromolecular amylose of 160,000 molecular weight, but amylase II decomposed this amylose less than soluble starch. The decomposition levels of a low-molecular-weight amylose (2,900) by amylases I and II were about half that of soluble starch. However, amylase III did not decompose the low-molecular-weight amylose, revealing differences among the enzymes in their actions on a small amylose. Although neither amylase I nor amylase II decomposed pullulan at all, amylase III did. Whether amylase III decomposed pullulan by acting on the α-1,4 bond or α-1,6 bond of the maltotriose remains a future subject to study. Amylase III seemed to differ in substrate recognition from amylases I and II. The authors plan to study the mechanism for pullulan decomposition by amylase III in the future. None of the enzymes decomposed such cyclic oligosugars as α- and β-cyclodextrins, and the enzymes seemed to recognize non-reducing-end substrates.

**Km and Vmax/Km.**

The Km value for amylases I, II, and III to starch was 0.33-0.42 mg/ml, with amylase II having higher affinity for starch than amylases I and III. The affinity levels toward high-molecular-weight amylose by amylases I and II were higher than toward low-molecular-weight amylose; Vmax/Km for amylases I and II toward amylopectin was the highest, and Vmax/Km of amylases III toward high-molecular-weight amylose was higher (Table 5).

**SUMMARY**

Amylases were extracted from germinating seeds of a waxy wheat. The enzymes were purified by ion-exchange chromatography, chromatofocusing, and gel chromatography to yield three purified enzymes, amylases I, II, and III. Amylases I, II, and III had optimum pH values of 5.5, 6.0, and 6.5, respectively, and were stable in a pH range of 4.5-7.0. The optimum temperature was 50°C for all three amylases. The molecular weights were 56,000 for amylase I, 64,000 for amylase II, and 53,000 for amylase III; the respective isoelectric points were pl 7.2, pl 5.4, and pl 4.8. All three amylases were SH enzymes, in that their
activities were inhibited by mercury and PCMB. Ni$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ also inhibited all the amylases. The decomposition products revealed amylase I to be a $\beta$-amylase, amylase II to be an $\alpha$-amylase, and amylase III to be a maltopentaose-forming enzyme. Both amylases I and II acted vigorously on amylopectin, this being a characteristic of waxy wheat amylase. Amylase III did not act much on amylopectin, but decomposed pullulan.

ACKNOWLEDGMENTS

The authors thank the Tohoku Agricultural Experiment Station for kindly providing the waxy wheat sample for this study.

LITERATURE CITED


Table 5. $K_m$ values and $V_{max}/K_m$ for the germinated waxy wheat amylases

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ value (mg/ml)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylase I</td>
<td>Amylase II</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>Amylose(MW160,000)</td>
<td>0.35</td>
<td>0.65</td>
</tr>
<tr>
<td>Amylose(MW2,900)</td>
<td>1.42</td>
<td>1.72</td>
</tr>
</tbody>
</table>

1 The enzyme was incubated with various concentrations of substrates (0.05-2mg) at 40°C for 10 min, and the data obtained were used to calculate the $K_m$ value and $V_{max}/K_m$ ratio by Lineweaver-Burk plots.
モチコムギ発芽種子アミラーゼの性状

谷口（山田）亜樹子１，２，菊池修平２，高野克己２
(１鎌倉女子大学，２東京農業大学)

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モチコムギ発芽種子からアミラーゼを抽出し，イオン交換クロマトグラフィー，クロマトフォーカシングおよびゲルクロマトグラフィーにて精製を行い，アミラーゼⅠ，アミラーゼⅡおよびアミラーゼⅢの３種類の精製酵素を得た．アミラーゼⅠ，ⅡおよびⅢの最適 pH は，pH 5.5，6.0 および 6.5 であり，各酵素の活性は pH 4.5 ～ 7.0 で安定であった．最適温度は，アミラーゼⅠ，Ⅱ，Ⅲともに 50℃であった．分子量は，アミラーゼⅠは 56,000，アミラーゼⅡは 64,000，アミラーゼⅢは 53,000 であり，等電点は pH 7.2，pH 5.4 および pH 4.8 であった．各酵素ともに，水銀，PCMB で活性が完全に阻害され，SH 酵素であり，また，Ni²⁺，Cd²⁺，Cu²⁺，によっても阻害された．分解生成物からアミラーゼⅠは β-アミラーゼ，アミラーゼⅡは α-アミラーゼ，アミラーゼⅢはマルトペンタオース生成酵素であり，アミラーゼⅠ，Ⅱともにアミロペクチンに最もよく作用し，モチ小麦アミラーゼの特性を示した．アミラーゼⅢは，アミロペクチンに対する作用性は低く，プルランを分解した．

キーワード：モチコムギ，アミラーゼ，性状，発芽種子