J. Microbiol. Biotechnol. (2008), **18**(4), 730–734

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effects of Calcium Ion Concentration on Starch Hydrolysis of Barley α-Amylase Isozymes

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Received: October 5, 2007 / Accepted: November 19, 2007

Barley α-amylase genes, *amy1* and *amy2*, were separately cloned into the expression vector of pPICZαA and recombinant *Pichia* strains were established by homologous recombination. Both AMYs from *Pichia* shared almost identical hydrolysis patterns on short maltooligosaccharides to result in glucose, maltose, or maltotriose. Against insoluble blue starch, AMY1 showed the highest activity at 0.1–5 mM calcium concentration, whereas 15–20 mM was optimal for AMY2. On the hydrolysis of soluble starch, unexpectedly, there was no significant difference between AMYs with increase of calcium. However, the relative activity on various starch substrates was significantly different between AMYs, which supports that the isozymes are clearly distinguished from each other on the basis of their unique preferences for substrates.

Keywords: Barley α-amylases, *Pichia* expression, calcium dependency, starch hydrolysis

Alpha-amylases (α-1,4-D-glucan glucanohydrolase; E.C. 3.2.1.1) are retaining enzymes of glycoside hydrolase family 13 acting on internal α-1,4-glycosidic linkages in starch materials [2, 10, 22]. These enzymes occur widely in bacteria, fungi, animals, and plants. Specifically, cereal α-amylases play an important role during seed germination *via* hydrolysis of the storage starch granules in the endosperm. Barley (*Hordeum vulgare*) malt contains two α-amylase isozymes, AMY1 and AMY2, readily distinguished by their isoelectric points (AMY1, low pI=4.7–4.9, AMY2, high pI=5.9–6.1) [3, 7]. Barley amylases are synthesized in the seed aleurone layer during germination and are speculated to play different roles in starch mobilization [3, 17]. Although barley AMYs share up to 80% amino acid sequence identity and almost identical three-dimensional structures, their enzymatic properties differ remarkably. AMY1 has higher affinity for calcium ions than AMY2 [1, 9, 18], and only AMY2 is strongly inhibited by the endogenous barley α-amylase/subtilisin inhibitor (BASI) [12, 15, 19, 21]. In addition AMY2 dominates in barley malt, but is poorly expressed in *Saccharomyces cerevisiae* and *Pichia pastoris* compared with AMY1 [4, 20]. Despite the well-known three-dimensional structures of both AMYs [5, 6, 13, 14] and a large amount of available biochemical data, the features that cause the major differences in isozyme properties remain unclear. Therefore, the comparative investigation of these isozymes may be one good method to elucidate the relationships between the structure and function of proteins. In this study, we tried to produce both recombinant isozymes with a six-histidine fusion tag in *P. pastoris* for the investigation of their enzymatic properties. After the production and purification of AMYs, their substrate specificities and calcium-dependent activities on various starch substrates, in addition to insoluble blue starch substrate, were determined.

Barley α-amylase isozymes, AMY1 and AMY2, are common plant-originated amylases expressed from barley malt. Barley cDNA clones E [18] and pM/C [17] were previously established by Rogers *et al.* and both genes were PCR-amplified and transferred into pUC19 plasmid vector, which were designated as pUCAMY1 and pUCAMY2, respectively. In order to construct *Pichia* expression vector containing AMY1, PCR amplification was performed with both primers PAMYN (5'-TTTGAATTCCACCAAGTC-CTCTTTCAGGGGTT-C-3') and PAMY1HC (5'-TTTTC-TAGAAGCTTAATGGTGATGGTGATGGTGCTCGAGG-CTCCGTTGT-AGTGTTGCCGC-3'), and pUCAMY1 as a template DNA. In the case of AMY2, all the components for PCR were identical to those for AMY1, except for the

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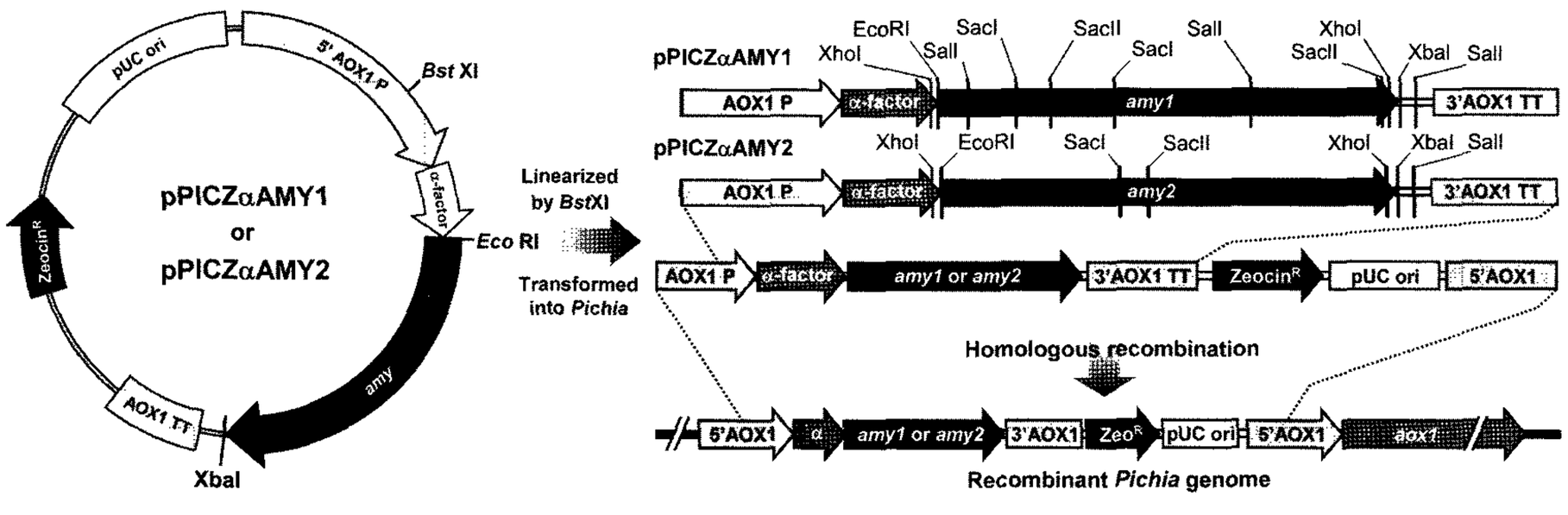

Fig. 1. Schemes for the construction of pPICZαAMY and the homologous recombination in *Pichia pastoris*.
Barley AMY1 and AMY2 genes were cloned into pPICZαA, which were designated as pPICZαAMY1 and pPICZαAMY2, respectively. Each recombinant plasmid was transformed and integrated into *Pichia* chromosome *via* homologous recombination.

template DNA (pUCAMY2), and the 3'-primer of PAMY2HC (5'-TTTTCTAGAAGCTTAATGGTGATGGTGATGGTG-CTCGAGTATTTTCTCCCATACGGCATAG-3'). Each AMY gene, *amy1* or *amy2*, was successfully amplified using Pyrobest polymerase (Takara Biomedicals Inc., Otsu, Japan). The PCR fragment was digested with EcoRI and XbaI and ligated into a *Pichia-E. coli* shuttle vector, pPICZαA (Invitrogen Co., Carlsbad, CA, U.S.A.). As shown in Fig. 1, the resulting constructs harboring *amy1* and *amy2* were obtained and designated as pPICZαAMY1 and pPICZαAMY2, respectively. Recombinant plasmids containing AMY1 or AMY2 gene fused with yeast-originated secretory peptide (α-factor) to transport any fused heterologous protein out of the cell membrane.

For the generation of recombinant *Pichia* strains [8, 23], pPICZαAMY1 or pPICZαAMY2 was linearized by BstXI treatment and transformed into *Pichia pastoris* GS115 (Invitrogen) by electroporation (2000 V, 25 μF, 200 Ω; GenePulser Xcell, Bio-Rad Co., Hercules, CA, U.S.A.). In a host cell, linearized recombinant DNA fragment was integrated into the *Pichia* chromosome *via* homologous recombination (Fig. 1). Recombinant *Pichia* cells, harboring the *amy1* or *amy2* gene, were directly screened on MMS (1.34% YNB, 2% glucose, 0.4 μg/ml D-biotin, 0.5% methanol, 100 μg/ml zeocin) agar plates by iodine staining assay on the basis of their starch hydrolyzing activity. As a result, both recombinant AMYs, fused with an N-terminal α-factor and a C-terminal six-histidine tag, were successfully expressed in *Pichia pastoris*.

In order to produce AMYs, recombinant *Pichia* was inoculated and cultivated in BMMY (1.34% YNB, 0.4 μg/ml D-biotin, 0.5% methanol, 1% yeast extract, 2% peptone, 0.1 M potassium phosphate, pH 6.0) medium over 120 h at 30°C. Productivity of recombinant AMY1 was over 30 times higher than that of AMY2. This result coincides with the previous reports for barley amylase isozymes [4]. However, the histidine-tag of both AMYs did not work for the affinity purification using an Ni-NTA column chromatography. Therefore, AMYs were precipitated mainly in the presence of 30–50% (w/v) ammonium sulfate. After the desalting against 20 mM Tris-HCl buffer (pH 7.0) by dialysis, the purification steps were performed *via* anion-exchange chromatography using Hi-Trap Q FF and Resource 15Q (Amersham Biosciences Co., Piscataway, NJ, U.S.A.). The molecular mass of purified AMY1 or AMY2 was estimated to be about 45,000 daltons by SDS-PAGE, which is similar to those from barley malt.

In order to investigate the hydrolysis patterns of AMYs, maltooligosaccharides from maltose (G2) to maltoheptaose (G7) were reacted with AMY1 and AMY2, respectively. After incubation at 37°C for 12 h, the reactions were stopped by boiling for 5 min and the reaction products were then analyzed by thin-layer chromatography (TLC) using Whatman K5F silica gel plates (Düsseldorf, Germany). There was no difference in hydrolysis patterns between AMYs, and both enzymes could be characterized as typical endo-acting α-amylases. Therefore, it was thought that both AMYs show similar action patterns on the resulting small oligosaccharides after the initial degradation of any starch polymers. Maltooligosaccharides, including G4 to G7, were readily hydrolyzed to glucose, maltose, or maltotriose by both AMYs, whereas maltose and maltotriose were rarely hydrolyzed to other small products such as glucose or maltose (Fig. 2). Accordingly, both barley AMYs possess the hydrolyzing activity towards starch polymers and oligosaccharides longer than maltotriose. In the case of maltotetraose, maltose was mainly produced by cleaving its central glycosidic linkage. Enzymatic stability of the AMYs at low pH of around 3.5 was compared. The activity of AMYs was measured by DNS (3,5-dinitrosalicylic acid) reducing sugar assay [11] against 1% soluble starch as a substrate. AMY1 was more stable at low pH than AMY2. The activity of AMY2 rapidly decreased to 51% after 3 min of incubation at pH 3.5. In spite of high similarity in the primary and tertiary structures between AMY1 and AMY2, subtle differences in structural aspects

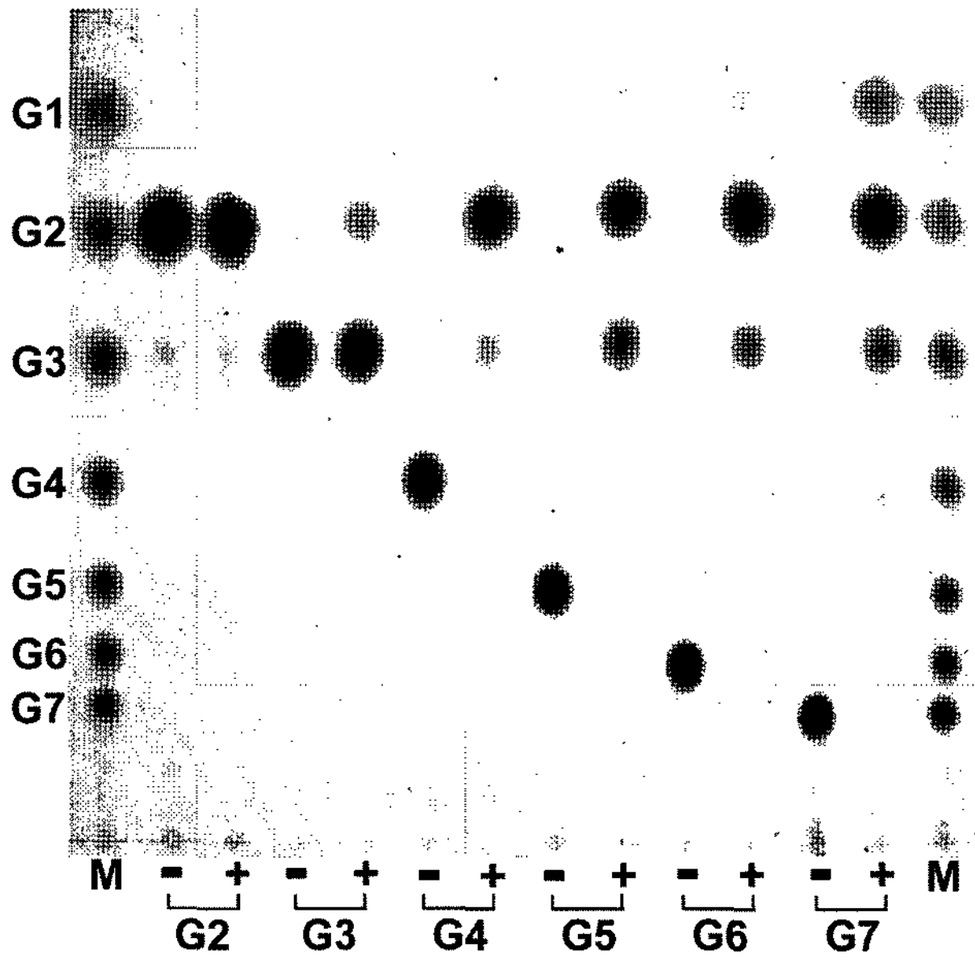

Fig. 2. Hydrolysis patterns of AMY1 on various maltooligosaccharides.
Action patterns of AMY1 against 1% maltooligosaccharides were analyzed by TLC (Whatman K5F plate, isopropanol:ethylacetate:water=3:1:1): lane M, 0.1% standard from glucose to maltoheptaose; G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose. Marks of (–) and (+) indicate the reactants without and with enzyme, respectively.

are likely to cause the significant discrepancy between them. This tendency was completely identical to those from barley malt and recombinant yeasts [16], which indirectly demonstrated that the characteristics of each recombinant AMY was not affected by the extra histidine residues.

The calcium-dependent hydrolyzing activity of AMYs was investigated against various starch materials. To measure the effect of calcium concentration on AMY1 and AMY2 activity, each AMY was reacted at 37°C with each substrate at various calcium concentrations from 0.1 to 50 mM, and then the starch hydrolyzing activity was determined by either insoluble blue starch [9] or DNS assay [11]. On insoluble blue starch (Amersham Biosciences), AMY1 showed the highest activity at 5 mM calcium concentration and maintained its high activity at a broad range from 0.1 to 10 mM of calcium ion. On the other hand, AMY2 showed the highest activity at 15–20 mM of calcium, compared with the lowest 20% of activity at 0.1 mM of calcium concentration. In brief, AMY1 showed optimum activity at low calcium ion concentration, whereas AMY2 did so at relatively high calcium concentration (Fig. 3A). These results are almost identical to those from barley malt and recombinant yeasts [20]. However, there was no significant difference in hydrolyzing activity on the soluble starch substrate (Showa Chemical Co., Tokyo, Japan) between AMY1 and AMY2, with increase of calcium concentration. As shown in Fig. 3B, the increase in calcium concentration

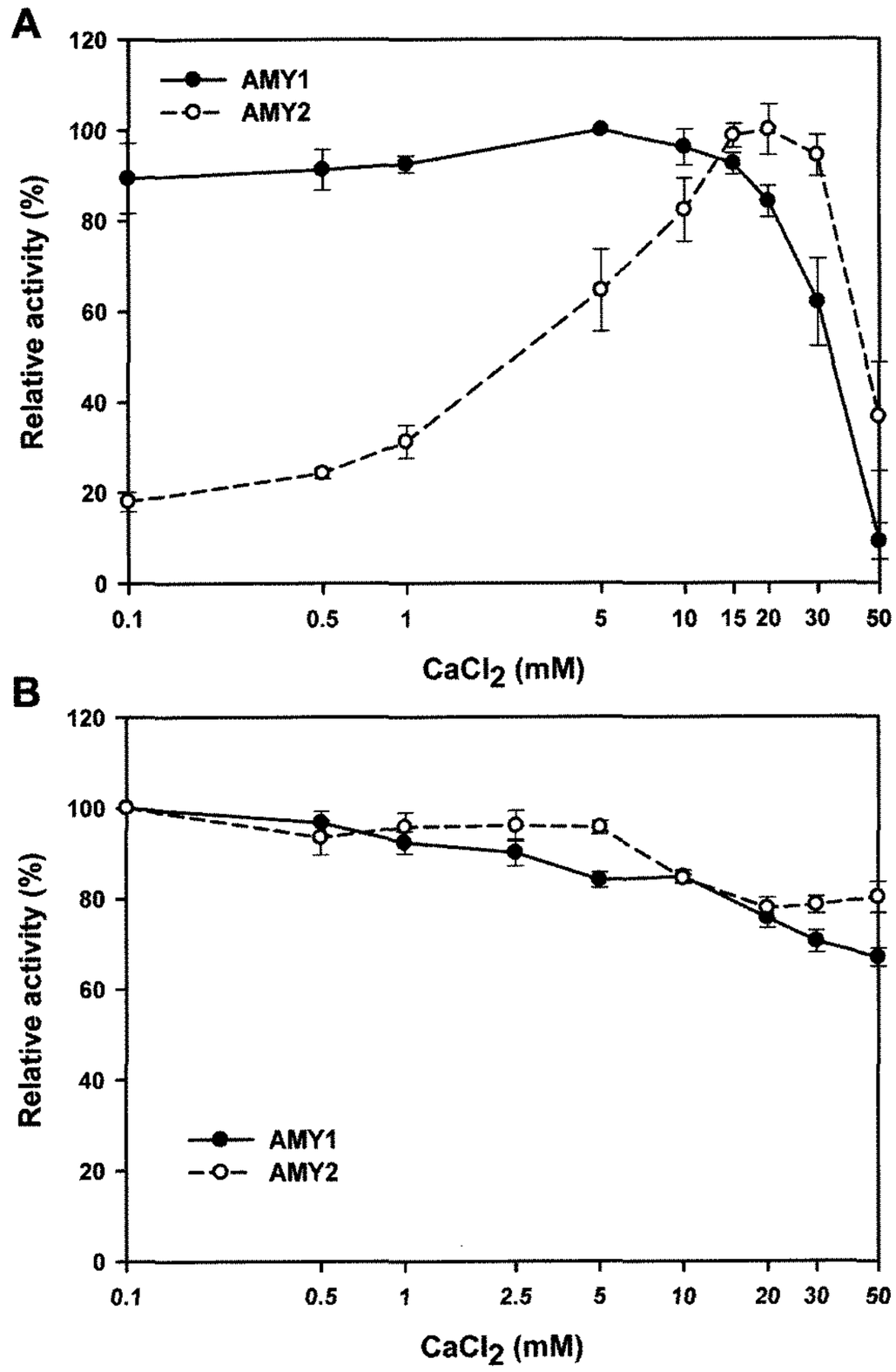

Fig. 3. Calcium-dependent activity of AMY1 and AMY2 on starch substrates.
Relative activity of AMYs was compared on (**A**) insoluble blue starch and (**B**) soluble starch from Showa Chemical Co. with increase of calcium concentration. The DNS assay method was used to determine the activity against soluble starch. The concentration of calcium ion is demonstrated by the logarithmic scale at the X-axis.

up to 50 mM caused the decrease by 20–30% in the activity of both AMYs. The calcium-dependent activities against other starch materials showed similar patterns as soluble starch and no significant difference between AMYs. It means that the remarkable calcium-dependent activity of AMYs may be resulted from the unique features of insoluble blue starch, one of the commercially modified starch materials. To date, both AMY1 and AMY2 were easily distinguished from each other, based on the differences in isoelectric points (pI) and calcium dependency. The researchers have focused on the calcium dependency on the basis of blue starch as a substrate. However, the experimental results in the present study suggest that the calcium dependency of AMYs can be closely related to the structural or chemical properties of starch substrates, which will be investigated further.

Table 1. Comparison of the hydrolyzing activities of barley α-amylase isozymes AMY1 and AMY2 on various starch substrates in the absence of calcium ion.

Starch substrate[a]	Specific activity (U/mg)[b]		Activity ratio of AMY1/AMY2
	AMY1	AMY2	
Insoluble blue starch	25.74±0.54	17.64±1.65	1.46
Corn starch	132.64±9.98	131.71±8.01	1.01
Potato starch	189.31±8.42	222.34±7.18	0.85
Soluble starch I	153.28±7.96	272.68±16.05	0.56
Soluble starch II	296.60±9.07	210.24±14.71	1.41

[a]Various starch substrates were commercially available and listed as follows: insoluble blue starch (customer's preparation from Amersham Biosciences Co.), corn starch and potato starch (Junsei Chemical Co.), soluble starch I (Showa Chemical Co.), and soluble starch II (Sigma-Aldrich Inc.).
[b]Specific activity on each starch substrate, except insoluble blue starch [9], was determined by the DNS reducing sugar assay method [11]. One unit is defined as the amount of enzyme that gives an increase in A_{620} (for insoluble blue starch assay) or A_{550} (for DNS assay) of 1 for a minute.

Even though the calcium ion may cause any structural changes for any specific starch polymer, both the action pattern and substrate specificity of AMYs can still differentiate them from each other. Therefore, the differences in substrate specificity between AMY1 and AMY2 were investigated against different starch substrates. In the absence of calcium ion, all the enzyme activities were measured and summarized in Table 1. Whereas soluble starch from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.) is the best substrate for AMY1, AMY2 prefers soluble starch from Showa Chemical Co. to the other starch substrates. As expected, the activity ratio of AMY1/AMY2 against blue starch was the largest at extremely low calcium concentration. AMY2 showed much higher activity on soluble starch from Showa Chemical Co. than AMY1. Accordingly, this result suggests that barley AMYs show significantly different substrate specificities, but the significance of calcium dependency in starch hydrolysis of AMYs may depend on the structure or the preparation of starch substrates.

Acknowledgment

This work was supported by Chungbuk National University Grant in 2005 and by the Danish Natural Science Research Council.

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