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MODIFICATION OF SUBSITE LYS RESIDUE INDUCED A LARGE INCREASE IN MALTOSIDASE ACTIVITY OF TAKA-AMYLASE A

Mikihiko Kobayashi*, Masami Miura and Eiji Ichishima

*National Food Research Institute, Tsukuba, Ibaraki 305,
Department of Agricultural Chemistry, Faculty of Agriculture,
Tohoku University, Sendai 981, Japan

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SUMMARY: A cross-linked modification of Taka-amylase A (TAA) by o-phthalaldehyde gave two enzymes, M_1- and M_2-TAA, which had optimum pH lower than that of native TAA by 0.5 to 1.0 pH units. The modified enzymes showed higher maltosidase activity, and produced glucose even at the initial period of hydrolysis, in contrast to the native TAA. The modification caused more than a 500-fold decrease in the k_0 value of native TAA for α-amylase activity, but a definite increase in k_0 value of 109.1 min^{-1} (native TAA) to 460.0 min^{-1} (M_1-TAA) and 147.1 min^{-1} (M_2-TAA) for maltotriose was evidence for improvement of maltosidase activity of modified enzymes.

Chemical modification of Taka-amylase A (TAA), a typical α-amylase produced by Aspergillus oryzae (1), has provided useful information on the role of amino acids located at the active site (2-4). Previously (2) we showed that a fluorescent reagent o-phthalaldehyde (OPA) was useful to form a cross-linking through the Lys and Cys residues of TAA, and the modification of Lys 209 located at subsite number 6 in the active site caused a significant change in the pI value, electrophoretic mobility, and affinity for the substrate soluble starch. We report changes in the enzymic properties of OPA-modified TAA, and improvement of maltosidase activity.

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MATERIALS AND METHODS

Taka-amylase A (TAA) from Aspergillus oryzae was a product of Sankyo Co. and purified according to the method described by Hase et al. (5). TAA was chemically modified by the fluorescent reagent o-phthalaldehyde as described previously (2). The OPA-modified TAA was subjected to a DEAE-Sephacel column and two modified enzymes, M_1-TAA and M_2-TAA were eluted by 0.25 M and 0.40 M NaCl (0 to 0.6 M gradient), respectively.

Polyacrylamide gel electrophoresis was done as before (2). Rm values were 0.57 and 0.63 for M_1- and M_2-TAA, respectively, and Rm 0.46-0.49 for native TAA (N-TAA). To determine enzyme activity production of reducing sugar was measured by the neocuproine method, and that of glucose was measured using a glucose oxidase kit (2).

The substrates, soluble starch and phenyl α-maltoside, were purchased from Merck Co. and Wako Pure Chemical Industries Ltd., respectively. Maltotriose and γ-cyclodextrin were the products of Nihon Shokuhin Kako Co.

RESULTS AND DISCUSSION

Optimum pH of N-, M_1-, and M_2-TAA. Upon the OPA modification, the polysaccharide hydrolyzing activity of M_1- and M_2-TAA decreased to 1/384 and 1/103 of that of N-TAA, determined by measuring the α-amylase activity with soluble starch. The three enzyme activities had the same initial velocity when oyster glycogen was used as a substrate at pH 5.2. When the action on the soluble starch was examined OPA-modified enzymes, M_1- and M_2-TAA, showed rather lower and higher activities than N-TAA, respectively (Fig. 1). Although the optimum pH of N-TAA was 5.5 - 6.0, that of M_1- and M_2-TAA was much lower being pH 4.5 - 5.0.

The activity of TAA on low-molecular-weight substrates such as phenyl α-maltoside, generally called as maltosidase activity, was also evaluated. The maltosidase activity was measured by the glucose oxidase method. M_1- and M_2-TAA had activity similar to that on soluble starch, whereas N-TAA hydrolyzed maltotriose poorly (Fig. 1b). Moreover, optimum pH of N-TAA for maltotriose (maltosidase activity) was around pH 5.0, which was shifted by 1.0 pH unit from the soluble starch (α-amylase activity).

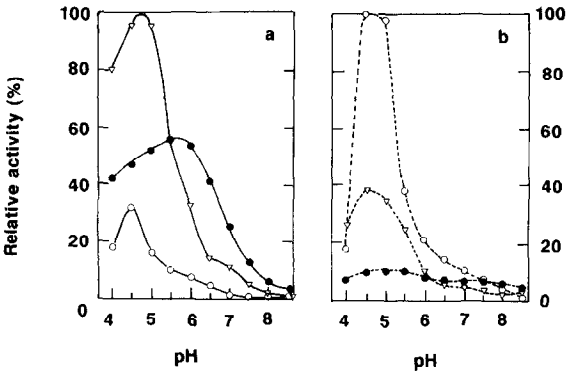

Fig. 1. Optimum pH of the native and modified TAA. (a) Relative activity was assayed with the substrate, soluble starch (0.04%) by the neocuproine method. (b) Relative activity was assayed with the substrate, maltotriose (30 mM) by the glucose oxidase method. N-TAA (●), M_1-TAA (○), and M_2-TAA (▽).

A substrate-dependent shift of the optimum pH was shown for the porcine pancreatic α-amylase (7). The optimum pH for activity on soluble starch (α-amylase activity) of this enzyme (pH 6.9) was shifted to pH 5.2, which was assayed with p-nitrophenyl α-D-maltoside (maltosidase activity). Kinetic and thermodynamic analysis indicated that the productive binding mode covering the fifth subsite changed the configuration between the catalytic residues and the glucosidic bond hydrolyzed, and modulated kinetic parameters depending on pH (7).

Hydrolysis of low-molecular-weight substrates. The activity of the OPA-modified enzymes was examined using maltotetraose as the substrate. While M_2-TAA gave much higher hydrolysis of soluble starch than M_1-TAA, the activity of M_2-TAA on maltotriose (Fig. 1b) and maltotetraose (Fig. 2a) was lower than that of M_1-TAA. Moreover, N-TAA gave no hydrolysis product even after overnight digestion. Since the time course of hydrolysis was measured by the glucose oxidase method, N-TAA was considered to produce no glucose from the substrate maltotetraose. N-TAA was also demonstrated to

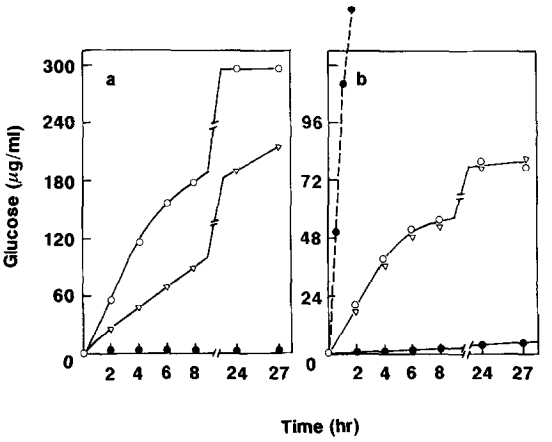

Fig. 2. Course of hydrolysis of low-molecular-weight substrates by the native and modified TAA. (a) Hydrolysis of maltotetraose (10 mM) was measured by the glucose oxidase method. (b) Hydrolysis of phenyl α-maltoside (10 mM) was measured by the neocuproine method. N-TAA (●), M_1-TAA (○), and M_2-TAA (▽). In (b), the concentration of N-TAA (●) was increased to 1000-fold (---).

have low hydrolyzing activity by the use of the synthesized substrate, phenyl α-maltoside (Fig. 2b). However, at a 1000-fold higher N-TAA concentration phenyl α-maltoside was hydrolyzed rapidly when measured by the increase in reducing sugar. In spite of the low activity of N-TAA, M_1- and M_2-TAA could attack the phenyl α-maltoside and gave mutually similar hydrolysis pattern (Fig. 2b).

These findings indicated that the OPA-modification of N-TAA produced enzyme having higher specificity to low-molecular-weight substrates than N-TAA. Maltosidase activity was also higher with TAA modified its His-residues by diethyl pyrocarbonate (3) and Lys-residues by phenyl azobenzoylchloride (4). However, in these case, maltosidase activity was not stimulated by the malto-oligosaccharides, which suggested that the hydrophobic structure of the phenyl group as an aglycon of the synthesized substrate might have a particular interaction with the modified-TAA. It was interesting to note that the OPA-modified TAA showed higher specificity to both phenyl derivative and malto-oligosaccharides.

Table I. Kinetic parameters of native and modified Taka-amylase A

Substrate	N-TAA		M_1-TAA		M_2-TAA	
	Km (mM)	k_0 (min^{-1})	Km (mM)	k_0 (min^{-1})	Km (mM)	k_0 (min^{-1})
Soluble starch	6.0×10^{-3}	5.4×10^{3}	9.1×10^{-3}	5.2	10.3×10^{-3}	10.1
Maltotriose	22.1	109.1	11.6	460.0	16.1	147.1
Phenyl α-maltoside	1.4	26.5	5.6	19.1	2.6	21.7

Kinetic parameters of modified TAA. Changes in the kinetic properties of the OPA-modified enzymes were evaluated by the use of high- and low-molecular-weight substrates. The k_0 values of M_1- and M_2-TAA for soluble starch were more than 500-fold smaller than that of N-TAA (Table I). By contrast, the k_0 value of M_1- and M_2-TAA was about 90- and 15-fold higher, respectively, when the activity was assayed with maltotriose instead of soluble starch. However, N-TAA had about 50-fold lower k_0 value with maltotriose. These findings clearly showed that OPA-modification caused the change of enzyme structure or conformation to reduce the α-amylase activity and enhance the maltosidase activity. Although the stimulation of maltosidase activity for phenyl α-glucoside was not clearly reflected in the kinetic parameters (Table I), Figs. 1 and 2 showed that M_1- and M_2-TAA have higher susceptibility to the phenyl derivative than N-TAA.

The extensive hydrolysis of soluble starch with N-TAA gave no glucose as the product, while M_1- and M_2-TAA gave both glucose and maltose as when maltotriose and maltotetraose were used as substrates (Figs. 1b and 2a). OPA modification of TAA might occurred at the sixth subsite, which has a strong subsite affinity of 2.9 kcal/mol next to the third subsite (4.5 kcal/mol)(8, 9). Cross-linked

OPA-modification at the sixth subsite may prevent the binding of substrates including oligosaccharides. Thus, maltotetraose, for example, was forced to bind at the subsite fifth to the second. Cleavage of the substrates occurred between the fifth and fourth subsite, and afforded glucose and maltotriose from maltotetraose. In conclusion, kinetic analysis of the OPA-modified TAA suggested that the Lys 209 located closely to the sixth subsite was modified and that this modification changed the enzymic properties of TAA.

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