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Macromolecule recognition of *Bacillus stearothermophilus* neopullulanase

Hiroshi Kamasaka, Kazuhisa Sugimoto, Hiroki Takata, Takahisa Nishimura & Takashi Kuriki*

Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502, Japan; tel.: ++ 81 6 6477 8425, fax: ++ 81 6 6477 8362, e-mail: kuriki-takashi@glico.co.jp

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Neopullulanase was a key enzyme in opening the door to clarification of the concept of the α-amylase family. The enzyme catalyzes both hydrolysis and transglycosylation of α-1,4- and α-1,6-glucosidic linkages at one active center. Different activities of neopullulanase toward amylose and amylopectin was noted previously. We recently found a unique way, using macromolecules, for recognition of the enzyme. A mixture of amylose and amylopectin from various sources was used as the substrate. Neopullulanase completely hydrolyzed amylose, but scarcely affected amylopectin. Although the molecular mass of amylopectin (approximately 10^8Da) decreased slightly, the degradation of amylopectin completely halted at the molecular weight of approximately 10^7 Da. This difference in action on two macromolecules was also found in cyclomaltodextrinase and maltogenic amylase from *Bacillus licheniformis*.

Key words: neopullulanase, cyclomaltodextrinase, maltogenic amylase, amylose, amylopectin.

Introduction

Starch constitutes most of the dry matter accumulating in the harvested organs of crop plants. It is not only the primary source of calories in the human diet, but also is regarded as a renewable resource in various industries. Starch is a mixture of two macromolecules; amylose and amylopectin. Amylose is essentially an α-1,4-linked glucose polymer. Amylopectin is an α-1,4-linked glucose-polymer branched by α-1,6-linkages. Various amylolytic enzymes have been described and used for starch-saccharifying industries (Yamamoto, 1988; 1995).

Pullulan is an extracellular α-D-glucan from *Aureobasidium pullulans* (Bender et al., 1959). The polysaccharide, with molecular mass of 60-240 kDa, consists mainly of maltotriose joined though α-1,6-glucosidic linkages interspersed with 5–7% of randomly-distributed maltotetraose units (Collins, 1998).

We found a new type of pullulan-hydrolyzing enzyme, neopullulanase (EC 3.2.1.135) from *Bacillus stearothermophilus* TRS40 (Kuriki et al., 1988), and proved that the enzyme catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic linkages (Imanaka & Kuriki, 1989), as well as transglycosylation to form α-1,4- and α-1,6-glucosidic link-

* Corresponding author

ages (TAKATA et al., 1992). The introduction of several replacements in the amino acid residues that constitute the active center of the neopullulanase provided the evidence that one active center of the enzyme participated in all four reactions described above (KURIKI et al., 1991). On the basis of a series of experimental results using the neopullulanase and structural similarities among the enzymes which catalyze these four reactions, we consequently refined and clarified a general idea for the enzyme family, the α-amylase family (TAKATA et al., 1992). By using ideas based on the concept of the α-amylase family (KURIKI & IMANAKA, 1999), we controlled the substrate preference and transglycosylation activity of the neopullulanase (KURIKI et al., 1996) and analyzed the regions which determined the specificity of maize branching enzyme (EC 2.4.1.18) isoforms (KURIKI et al., 1997).

We recently found a unique way to recognize the neopullulanase using macromolecular substrates (KAMASAKA et al., 2002). Although the neopullulanase completely hydrolyzed amylose to produce maltose as the main product, the enzyme scarcely hydrolyzed amylopectin. This clear difference in macromolecule recognition was also observed for the reaction of cyclomaltodextrinase (OHDAN et al., 2000) and *Bacillus licheniformis* maltogenic amylase (KIM et al. 1992). These three types of enzyme exhibited 40–60% amino-acid sequence identitiy (PARK et al., 2000). There is currently great interest in structure and function relationships among these enzymes (MACGREGOR et al., 2001). In this article, we review the distinct substrate preferences of the neopullulanase. The recognition of macromolecules by the enzyme is also discussed in terms of the structure-function relationship.

Comparison of the degradation of amylose or amylopectin with neopullulanase and other amylolytic enzymes

Potato amylose and amylopectin solutions were prepared and used as the substrate for the neopullulanase, α-amylases from *Bacillus liqueniformis* and *Bacillus subtilis*, and a β-amylase from soybean. Equal amounts of activity of enzymes on potato amylose were used. α-Amylases and the β-amylase efficiently hydrolyzed both amylose and amylopectin, and produced the same products from amylose and amylopectin. The neopullulanase efficiently hydrolyzed amylose to produce maltose and a small amount of glucose; the enzyme scarcely hydrolyzed amylopectin.

The different specificities of the neopullulanase on amylose and amylopectin was confirmed by using a mixture of a synthetic amylose, with an average molecular mass of 70 kDa, and potato amylopectin. Since these two components in the mixture were clearly fractionated by a column chromatography system, the two components could be quantitatively analyzed. The peak of the amylose gradually disappeared on the neopullulanase treatment, while the peak of amylopectin was not affected even after long incubation. These results indicated that the neopullulanase selectively hydrolyzed amylose even in the mixture of amylose and amylopectin.

Potato starch, containing about 20% amylose and 80% amylopectin, was used as a substrate for the neopullulanase. After an appropriate incubation period, amylose, amylopectin, and low molecular mass fractions were fractionated using 1-butanol (LANSKY et al., 1949). The amylose fraction gradually disappeared on treatment with the enzyme, but the amylopectin fraction was not significantly affected during the reaction period. The low molecular mass fraction increased in step with the decrease in the amylose fraction. The result was consistent with the results described above and again indicated that the neopullulanase selectively hydrolyzed amylose in starch.

The time-course of the effect on the molecular mass of amylose and amylopectin fractions during the reaction periods was analyzed by high-performance liquid chromatography with a multi-angle laser-light-scattering photometer and a differential refractometer. The molecular mass of amylose rapidly decreased below 10^5 Da within a short reaction period, and the amylose fraction could not be obtained by the method using 1-butanol. The amylose was completely hydrolyzed to maltose and glucose. The molecular mass of the native amylopectin from potato was more than 10^8 Da. Although the molecular mass of potato amylopectin decreased slightly to approximately 10^7 Da, it could not be lowered further by the neopullulanase reaction.

The chain-length distribution of amylopectin after the neopullulanase treatment was investigated. The amylopectin was debranched by isoamylase, and the products were analyzed on high-performance anion-exchange chromatography. Little difference between the neopullulanase-treated amylopectin and intact amylopectin could be found in the chain length distribution.

Similar substrate specificity of cyclomaltodextrinase, *B. licheniformis* maltogenic amylase and the neopullulanase

The initial velocities of the reactions of the neopullulanase and the other amylolytic enzymes with various substrates were analyzed. The same amount of each enzyme was used, on the basis of potato amylose-hydrolyzing activity.

Substrate specificities of cyclomaltodextrinase from *Bacillus* sp. A2-5a (Ohdan et al. 2000) and *B. licheniformis* maltogenic amylase (Kim et al., 1992) were similar to that of the neopullulanase. These three enzymes efficiently hydrolyzed amylose, maltooligosaccharides, and cyclomaltodextrins and scarcely affected amylopectin. On the other hand, α-amylases and β-amylase efficiently hydrolyzed both amylose and amylopectin and did not exhibit significantly different specificity toward these two starch components.

Structure-function relationship of the neopullulanase, cyclomaltodextrinase and *B. licheniformis* maltogenic amylase

The neopullulanase, cyclomaltodextrinase and *B. licheniformis* maltogenic amylase exhibit 40–60% amino-acid sequence identity (Park et al., 2000). They are intracellular enzymes without a signal sequence for secretion. On the other hand, α-amylases are usually extracellar enzymes. Sequence identities between the neopullulanase and α-amylases are less than 30% (Kuriki et al., 1996). The neopullulanase, cyclomaltodextrinase, *B. licheniformis* maltogenic amylase and α-amylase hydrolyze α-1,4-glucosidic linkages of amylose and produce α-anomeric products; therefore, they are configuration-retaining enzymes. They belong to the α-amylase family (Takata et al., 1992; Svensson, 1994; Kuriki & Imanaka, 1999). However, the neopullulanase, cyclomaltodextrinase and *B. licheniformis* maltogenic amylase are clearly different from α-amylase from the points of view of the specificity toward amylose and amylopectin, cellular location, and homology at the amino-acid sequence level. Indeed, the former three enzymes have a distinct domain composed of approximately 130 amino-acid residues preceding the catalytic domain (Kuriki et al., 1996; MacGregor et al., 2001). Although the role of this domain is as yet uncertain, it was suggested, from the results obtained from the crystal structure of maltogenic amylase of a *Thermus* strain (Kim et al., 1999), that the domain was involved in the specificity through its interaction with the active site region of the other monomer. The physiological role and the problem of classification of the enzymes of the α-amylase family need further investigation.

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