UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND CHARACTERIZATION OF THERMOSTABLE AMYLASES FROM BACILLUS CIRCULANS ISOLATED FROM A LOCAL HOT SPRING

SHARIFAH SHAHRUL RADIAH DT. SYED ALWEE

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PRODUCTION AND CHARACTERIZATION OF THERMOSTABLE AMYLASES FROM *BACILLUS CIRCULANS* ISOLATED FROM A LOCAL HOT SPRING

By

SHARIFAH SHAHRUL RABIAH BT. SYED ALWEE

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology, Universiti Pertanian Malaysia

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Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia as fulfilment of the requirements for the degree of Master of Science.

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SHARIFAH SHAHRUL RABIAH BTE SYED ALWEE

SEPTEMBER 1992

Chairman : Dr. Baharuddin Abdul Ghani

Faculty : Food Science and Biotechnology.

Two strains of amylolytic Bacillus were isolated from a hot spring in Negeri Sembilan and were identified as Bacillus circulans and designated as strains SB-1 and SB-21.

The optimal temperature and pH for growth and enzyme production by both strains were found to be at 55°C and pH 7.0, respectively. The enzyme was produced from the beginning of growth and reached maximum production at 72 hours. The production of amylase was partially induced and production occurred only in the presence of 1% starch. The presence of 20 mM maltose or maltotriose enhanced enzyme production. The production was found to be repressed by 20 mM glucose.

The crude enzyme preparation of SB-1 was purified through ion-exchange chromatography after 20-40% ammonium sulfate

precipitation and ultrafiltration. A single activity peak and 31.6% yield was obtained with a 53.9 fold purification. Using SDS-PAGE the enzyme was shown to be homogenous and the molecular weight of the purified amylase was estimated to be about 60,000 dalton. The optimum temperature and pH for the activity of the purified amylase were shown to be 70°C andd pH 5 - 9 respectively. The purified enzyme was less stable at higher temperature but 1 mM $CaCl_2$ stabilizes it significantly. The purified enzyme has higher affinity towards longer chain dextrins and more complex substrates such as starch. Thin-layer chromatography of enzymatic hydrolysis on various starches and dextrins indicated that the purified amylase behaves similar to that of α-amylase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains.

PENGHASILAN DAN PENCIRIAN AMILASE TERMASTABIL DARI *BACILLUS CIRCULANS* YANG DIPENCILKAN DARI KOLAM AIR PANAS

Oleh

SHARIFAH SHAHRUL RABIAH BTE SYED ALWEE

SEPTEMBER 1992

Pengerusi : Dr. Baharuddin Abdul Ghani

Fakulti : Sains Makanan dan Bioteknologi.

Dua strain bacteria penghasil amilase telah dipencilkan dari kolam air panas dari Negeri Sembilan dan dikenalpasti sebagai *Bacillus circulans* dan strain-strain ini telah dinamakan sebagai strain SB-1 dan SB-21.

Suhu dan pH optima untuk penghasilan amilase dan pertumbuhan mikroorganisma didapati pada 55°C dan pH 7.0. Enzim ini dihasilkan dari permulaan pertumbuhan dan mencapai penghasilan yang maksimum pada 72 jam. Amilase ini adalah enzim yang separa aruhan dimana penghasilan berlaku hanya apabila terdapat kanji 1%. Kehadiran maltosa dan maltotriosa 20 mM dapat meningkatkan lagi penghasilan enzim. Kehadiran glukosa didapati menindas penghasilan enzim.

Enzim daripada Bacillus circulans strain SB-1 kemudiannya ditulenkan melalui kromatografi turus pertukaran ion selepas pemendakan ammonium sulfat 20-40% dan penurasan ultra. Satu puncak aktiviti dan 31.6% hasil penulenan didapati dengan faktor penulenan sebanyak 53.9 kali ganda. Dengan menggunakan SDS-PAGE, enzim ini didapati homogen dan beberat molekul dianggarkan 60,000 dalton. Suhu dan pH optimum bagi enzim yang ditulenkan ini adalah 70°C dan pH 5 - 9. Walaubagaimanapun, enzim ini didapati kurang stabil pada suhu yang tinggi tetapi 1 mM $CaCl_2$ dapat menstabilkannya. Amilase yang telah ditulinkan ini mempunyai keafinan yang lebih tinggi terhadap dekstrin berantai panjang dan substrat kompleks seperti kanji. Kromatografi lapisan nipis bagi hidrolisis enzim terhadap berbagai jenis kanji menunjukkan bahawa amilase yang ditulenkan ini bertindak seperti α-amilase.

CHAPTER I

INTRODUCTION

Amylases are classified as 'hydrolases' (EC 3.2.1) and are enzymes that catalyze the hydrolysis of O-glycosyl compounds (Yamamoto, 1988). They are divided into three main types namely exo-acting, endo-acting or debranching enzymes according to their mode of action. Exo-acting amylases degrade amylose and amylopectin of starch by the successive removal of low molecular weight products from the non-reducing chain ends. Exo-acting amylase include β-amylase (Exo-α-1,4-glucan maltohydrolase, EC 3.2.1.2) and glucoamylase (Exo-α-1,4-glucan glucanohydrolase, EC 3.2.1.3) which produce maltose and glucose respectively (Fogarty and Kelly, 1980). Endo-acting amylase, which is the most widely occuring form of amylase, consists of only α-amylase (Endo-α-1,4-glucan glucanohydrolase, EC 3.2.1.1). It catalyzes the random hydrolysis of starch-type polysaccharides, which results in a rapid reduction of iodine blue value and viscosity of substrate depending on the specific number of bonds broken. Compared with β-amylase or glucoamylase, the reduction of molecular weight of substrate is much faster with α-amylase than either β-amylase or glucoamylase. Debranching enzymes which include pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) hydrolyse only α-1,6-glycosidic bonds.

α-Amylases are found in mammals, higher plants, fungi, and bacteria. Bacteria are the most widely used and versatile source of α-amylase. In addition, this source is easily amenable to genetic manipulation thus providing an opportunity for the development of enzymes with more desirable operational parameters. To date most of the α-amylases used in industry are produced by Bacillus sp., especially Bacillus amyloliquefaciens and Bacillus licheniformis. Bacillus sp. produce both saccharifying and liquefying α-amylases which are distinguishable by their mechanisms of starch degradation. For example, Bacillus subtilis var. amylosaccharíticus, Bacillus subtilis Marburg and Bacillus natto all produce saccharifying α-amylase (Matsuzaki et al., 1974). Bacillus amyloliquefaciens, on the other hand, produces large quantities of liquefying α-amylase (Welker and Campbell, 1967).

Thermostable amylases have been isolated from various strains of Bacillus spp. These include Bacillus stearothermophilus (Manning and Campbell, 1961), Bacillus licheniformis (Morgan and Priest, 1981), Bacillus coagulans (Medda and Chandra, 1980) and Bacillus acidocaldarius (Buonocore et al., 1976). Four interesting observations have been made on the comparative biochemistry of thermostable enzymes. First, the heat stability of these enzymes may be associated with increased hydrophobic bonding at the core of the molecule (Doig, 1974). Second, the thermostable α-amylase was found to have an unfolded structure as opposed to a more

typical globular structure of the mesophilic enzyme (Manning and Campbell, 1961). Third, an increase in hydrogen bonding may be involved in heat stability. Finally, these enzymes appear to lack cysteine residues and as a consequence cannot contain disulfide linkages (Amelunxen, 1967).

Alpha-amylases have numerous biotechnological applications, for example, in starch conversion processes α-amylase are used to produce syrups containing oligosaccharides, maltose and glucose, in the brewery, food and textile industries. Bioconversion of starch into sugars and other alternative food products makes up the major part of the starch-processing industry. In the industrial production of glucose syrup, gelatinized starch is subjected to hydrolysis by α-amylase to produce dextrins. This process is called 'liquefaction'. The dextrins are then hydrolysed by glucoamylase in the 'saccharification' process to produce glucose (Swinkel, 1986).

The enzymatic hydrolysis of starch is widely used because it offers many advantages over the older technology of acid conversion. Enzymes are more specific and efficient as catalysts when compared to acid. According to Swinkel (1985), the enzymatic process resulted in the following benefits : 1) by-product formation is reduced more than 10-fold; 2) the finished syrup only contain half the ash content; 3) less color formation and easier refining; 4) Na^+ and Cl^- level is reduced

5-fold; 5) lower quality starch can be used; and 6) lower energy costs.

This project was done mainly for academic reasons, to learn about local amylolytic thermophilic bacteria and the enzymes produced by them. The knowledge gained in this study would further contribute towards the utilization of local substrates by biotechnology.

CHAPTER II

LITERATURE REVIEW

Starch : The Substrate

Starch occurs as the major reserve carbohydrate in all higher plants in the form of water insoluble granules. It is readily assimilated in the human diet; in fact, a very high proportion of the world food intake is starch. Microscopic examination reveals that starch is composed of tiny, white granules, ranging from about 2 to 100 um in diameter. The size and shape of the granules are peculiar to each variety of starch (Table 1). Other than cellulose, starch is the next most abundant compound synthesized by plant cells. It is a 'renewable' substance; a new supply of starch is grown annually (Swinkel, 1985). The properties of starch vary with the plant source from which it is derived.

Starch is a polymeric carbohydrate, composed of C, H and O atoms in the ratio 6:10:5, $(C_6H_{10}O_5)_n$. It is considered to be a condensation of glucose polymers. The glucose units are present as anhydroglucose units. If starch is treated with acids or certain enzymes, it is broken down into its constituent glucose molecules. The glucose units are linked to one another through the C_1 oxygen as glucoside bond. The glucoside bonds are stable under alkaline conditions and hydrolyzable under acid

Table 1

Properties of Starch Granule

Starch	Type	Size Range (Diameter) (µm)	Shape
Corn	Cereal	3 - 26	Round, Polygonal
Potato	Tuber	5 - 100	Oval, Spherical
Wheat	Cereal	2 - 35	Round, Renticular
Tapioca	Root	4 - 35	Oval, Truncated
Rice	Cereal	3 - 8	Polygonal, Angular
Sago	Pith	5 - 65	Oval, Truncated

Source: Swinkels (1985)

conditions. The glucose unit at the end of the polymeric chain has a latent aldehyde group and is known as the reducing end group.

Most starches are a mixture of two types of polymers : (1) a linear chain molecule termed amylose and (2) a branched polymer of glucose termed amylopectin. Each of these polymers have a wide range of molecular sizes. Starches of different origin have different amylose and amylopectin ratios (Table 2). Amylopectin made up about 75-85% of starch.

Amylose, the linear polymer, contained up to 6000 glucose units and is connected by α-1,4-glucosidic linkages (Figure 1). Enzyme studies also indicated a trace amount of branching in

Table 2

Amylose + Amylopectin Contents of Various Starches

Starch	Amylose (%)	Amylopectin (%)	Ave. DP Amylose	Ave. DP Amylopectin
Corn	28	72	800	2,000,000
Potato	21	79	3000	2,000,000
Wheat	28	72	800	2,000,000
Tapioca	17	83	-	-
Rice	17	83	-	-
Sago	27	73	-	-
Waxy maize	0	100	-	-

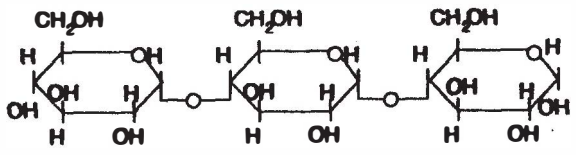

Figure 1. Structure of Amylose Molecules

the amylose molecule. The branched amylose may contain 3 to 20 chains, with an average chain length of about 500 glucose units. The degree of polymerization (DP) of amylose covers a wide range depending upon the source of the starch (Swinkel, 1985). For example, the amylose molecules of potato and tapioca starch have a substantially higher molecular weight than maize and wheat starch amylose.

Amylose forms inclusion complexes with iodine and various organic compounds such as butanol, fatty acids, surfactants, phenols and hydrocarbons. These complexes are insoluble in water. It is believed that amylose complexes by forming a helix coil around the complexing agent (Swinkel, 1985). The complex of amylose with iodine gave a characteristic blue colour, which is used to establish the presence of amylose-containing starch.

Amylopectin (Figure 2) is a highly branched structure consisting of short linear chains with a DP ranging from 10-60 glucose units, with an average of about 22. The glucose units are connected to each other by α-1,4- and α-1,6-glucosidic linkages. The glucose units with an α-1,6-glucosidic linkages are the branching points which cause the interlinkages of the glucose residues that gave rise to a ramified or bushlike structure of the amylopectin molecule. The branching points make up about 5% of the total glucose unit in amylopectin. Amylopectin is one of the largest molecules in nature with an average DP of about 2 million. The molecular weight of

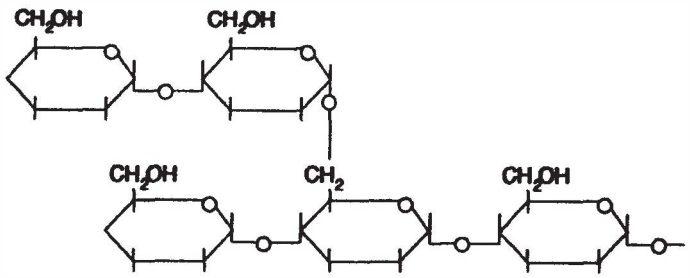

Figure 2. Structure of Amylopectin Molecules

amylopectin is about 1 000 times as high as the molecular weight of amylose (Swinkel, 1985).

Starch granules are insoluble in water below 50°C (Fogarty, 1983). When a suspension of starch in water is heated beyond a critical temperature, the granule will swell many times its original size. The critical temperature also known as gelatinization temperature, varies from 55°C to 80°C depending on the type of starch. When heating is continued, the swollen starch granules will disintegrate into a viscous paste which is the swollen starch aggregates. The process of transformation from starch into the viscous starch paste is called gelatinization. This hydrocolloidal property of starch makes it suitable for a great variety of applications. Starch and its derivatives are widely used in the manufacture of foods, paper, textiles, adhesive, pharmaceuticals and building materials. Half of starch produced is used to produce syrups and sugars.