CHAPTER 2

MATERIALS AND METHODS

I. Materials

"Look Pang"

Six samples of "Look Pang" were kept in the refrigerator through out the experiment. The sources of "Look Pang" and their intended uses were shown in Table 1.

Cultures used

Nine isolates of mold and six isolates of yeast isolated from six samples of "look Pang" (Sukhumavasi, 1973) were maintained on slants of yeast malt extract (YM) medium (Phaff et al, 1966) with occasional transfer (27, 22).

Media and chemicals

Culture media used were the product of Difco.

Maltotriose and maltotetraose were prepared by partial hydrolysis of amylose, followed by chromatography on a charcoal column by the method of Whelan et al (1953) (32). Maltohexaose, maltoheptaose and maltooctaose were prepared by enzymatic hydrolysis of α-, β- and γ-cyclodextrins, respectively, using a specific intracellular amylase (Kato et al, 1975) from Pseudomonas

sp. MS1. Maltitol, maltotriitol and maltotetraitol were obtained by the reduction of their corresponding sugars with sodium borohydride. Maltodextrin (DP 100) and pullulan were obtained from Hayashibara Biochemical Laboratory, Okayama. Reducing C^{14}- labeled maltodextrins were prepared by the reaction of C^{14}- glucose and α-cyclodextrin with amylase of Bacillus macerans (French et al, 1954). Other chemicals were commercial products of the highest purity available.

II. Methods

Determination of amylolytic activity of "Look Pang"

1. Preparation of enzyme extract

5 grams of "Look Pang" was extracted with 100 ml of distilled water for 60 minutes at room temperature. The aqueous extract was then filtered through the Whatman No. 1 paper.

2. Assay of Amylolytic activity

α-amylase activity was determined by the Modified Wohlgemuth method as described by Tauber (1950) (28). The brief procedure was as follows :

a. To each of 10 test tubes a 5 ml of 0.008% iodine solution was added.

b. Twenty ml of enzyme extract was added to 10 ml of 2%

solution and thoroughly mixed. The time was recorded as soon as the extract came into contact with the starch solution. After 5 minutes, 1 ml of the mixture was added to the first iodine tube, and the color occurred was observed. The next 5 or 10 minutes, another 1 ml of the mixture was added to the second iodine tube. This procedure was repeated at suitable interval of time until the colorless end point with iodine was reached. The exact dextrinization time was recorded and the α-amylase unit was calculated.

α-amylase unit is defined as the number of grams of soluble starch which, under the influence of an excess of β-amylase are dextrinized by one gram of enzyme containing sample in one hour at 30°C. The volume of enzyme solution used is expressed in terms of the weight of material extracted, and the volume of 2% soluble starch dextrinized in terms of the weight of starch it represents (28).

Comparison of amylase producing capacity of a single culture and mixed cultures isolated from certain "Look Pang"

A. Production of amylase

Fifteen cultures were separately grown aerobically on reciprocal shaker at room temperature in a 5 ml of YM broth, pH 5.5, for 24 hours. And then 2% of seed culture was inoculated into 100 ml YM broth, pH 5.5, in 250 ml Erlenmeyer flask. The

inoculated broth was incubated with shaking 1,500 rpm at room temperature for 50 hours.

B. Determination of amylase producing capacity

The cultured mash was filtered through the Whatman No. 1 paper and the filtrate was assayed for α-amylase activity by the method of Tauber (28).

Morphology and fermentation study of the amylolytic potent strain

Cell morphology study was made on 1Y strain, which considered to be the most amylolytic potent isolate. The study included the observation of the growth development in solid medium, liquid medium (YM agar and YM broth, respectively) as well as the slide culture on corn meal agar. Its pseudomycelium and spore formation were microscopically investigated.

The assay of sugar fermentation of 1Y strain was performed by using the Hugh and Liefson medium (Baker, 1967) (3).

Medium modification for formation of amylase

In order to obtain the proper medium for high enzyme synthesis including good growth of 1Y strain, the basal medium, YM was modified. The modification of the basal medium was on varying the kinds of saccharides used (Table No. 4) and the pH of the medium. Volumes of 95 ml of those media in 500 ml coni-

cal flasks were inoculated with 5 ml of seed culture which had been grown in the same medium. The inoculated flask was incubated on reciprocal shaker at 32°C for 50 hours. Growth was followed in Klett-Summerson colorimeter (590-660 mμ), and the activity was assayed as gram of reducing sugar released using the method of Nelson and Somgyi (1944) (17).

Production and purification of amylase

1. Production

The 2% by volume of 24 hours seed culture of 1Y strain was inoculated into the modified medium as described earlier. The volume of the medium was 20 liters in a 30 liters fermenter. After 50 hours the cells were removed by centrifugation.

2. Purification

All operations were carried out in 0.01 M acetate buffer, pH 5.5 at 4°C, unless otherwise stated.

The enzyme supernatant was brought to 50% saturation of $(NH_4)_2SO_4$ and the resulting precipitate which is another protein not the amylase was removed by centrifugation. More solid $(NH_4)_2SO_4$ was added to the supernatant to give 70% saturation. By centrifugation, the precipitate was collected, dissolved in 200 ml of buffer. The solution was dialyzed twice. The first was in running water for 16 hours, the second was in 5 liters

of the buffer for 24 hours. The precipitate formed during dialysis was removed and the dialyzed enzyme was applied to a [1]DEAE cellulose column (5 x 15 cm.). The column was washed with the buffer and eluted with 500 ml of a linear gradient of NaCl (0-0.5 M) in the buffer. During the fractionation process, the amylase activity and the protein content of enzyme fractions were determined. Amylase was eluted as one major peak in 0.25-0.35 M NaCl fractions, with a small minor peak (Figure 4a). Fractions of the major peak were combined. The NaCl was removed by concentrating the enzyme solution to 3 ml by ultrafiltration through a collodion membrane under vacuum. Then the solution was fractionated through a Sephadex G-200 column (3 x 45 cm.). The column was eluted with the buffer. Amylase activity emerged as a single symmetrical peak coinciding with the protein (Figure 4b). The fractions in this peak were combined. The enzyme was tested for purity by gel electrophoresis. The molecular weight of the enzyme was estimated by using a Sephadex G-200 column and comparing its elution volume with those of a series of known molecular weights.

The purified enzyme was kept in the buffer at 4°C for further experiment.

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[1]DEAE - cellulose - Diethylaminoethyl cellulose.

Determination of protein concentration

Protein concentration was determined by method of Lowry et al (1951) using bovine serum as a standard (15), and by using a Hitachi, 124 UV-Vis-Spectrophotometer, absorbance at 280 mμ.

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out at pH 8.3 by the method of Davis (1964) with a current of 2 mA/tube for 3.5 hours. The gels were stained with Naphthol blue black [1]C.I. No. 20470 (5).

Properties of amylase

A. Effect of pH and temperature on the activity and stability of amylase

Activity and stability of amylase at various pH from 3.5-10.0 was studied. The three buffer solutions used were 0.1 M acetate buffer for pH 3.6-5.6, 0.1 M phosphate buffer pH 5.7-8.0 and 0.1 M ammediol buffer pH 8.0-10.0. For examination of pH stability, enzyme solution of various pH values were incubated for 30 minutes at 40°C, and then adjusted to pH 5.5.

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[1]C.I. - the Color Index of the E. Merck AG. Darmstadt, Germany.

The remaining amylolytic activity was measured by the standard method (Nelson & Somogyi). For the examination of temperature stability, the enzyme in 0.1 M acetate buffer solution, pH 5.5, was incubated for 30 minutes at various temperature ranging from 10° to 80°C. The solution were cooled rapidly to 4°C, and the remaining activities were examined.

B. Substrate specific activity of amylase

Samples of 2 mg each of potato starch, amylose, amylopectin and glycogen were incubated with the enzyme in the buffer in a total volume of 1 ml at 40°C and at intervals the end products were detected by paper chromatography. Chromatograms was developed by the descending method with n-butanol-pyridine-water (6:4:3) as solvent. The reducing sugars were detected with alkaline silver nitrate reagent (Figure 7 and Figure 8).

The rate of reactions on various substrates were measured quantitatively by the initial velocities. Substrates were prepared in 0.1 M acetate buffer, pH 5.5, at a final concentration of 1% solution for macromolecular polysaccharides and 0.002 M for oligosaccharides. A suitable quantity of enzyme was added to produce a linear increase in the reducing value of glucose releasedduring the first 15 minutes of reaction time. The reducing power was determined by the method of Nelson and Somogyi (17). The true glucose was measured by the method of Saifer (1958) (24).

C. Reaction mechanism of amylase

The reaction mechanism on the substrate of amylase was studied by autoradiography. The radioautograms were examined by the two dimensional paper chromatographic technique of Pazur and Okada (1966), using the maltodextrin labeled at the reducing end (20).