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ACTION OF AMYLASE INHIBITORS PRODUCED BY *Streptomyces* sp. ON SOME CARBOHYDRATE HYDROLASES AND PHOSPHORYLASES*†

SEINOSUKE UEDA, YOJIRO KOBA, AND HIROTO CHAEN

Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, Fukuoka (Japan)

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ABSTRACT

The amylase inhibitor produced by *Streptomyces* sp. has been separated into four fractions (inhibitors A, B, B′, and C) that were homogeneous by paper chromatography and paper electrophoresis. These inhibitors acted not only on glucoamylase and alpha amylase, but also on α-D-glucosidase, cycloamylose glucanotransferase, and phosphorylase. The amylase inhibitors A and B slightly activated yeast α-D-glucosidase.

INTRODUCTION

The occurrence of substances showing inhibitory action on amylases has been demonstrated in a variety of plants, including germinated buckwheat[1], wheat[2], rye[3], some of the sorghums[2,3], and resting potato tubers[4]. Some of these inhibitors of plant origin have been investigated with regard to their purification and characterization, but little is known about inhibitors produced by microorganisms.

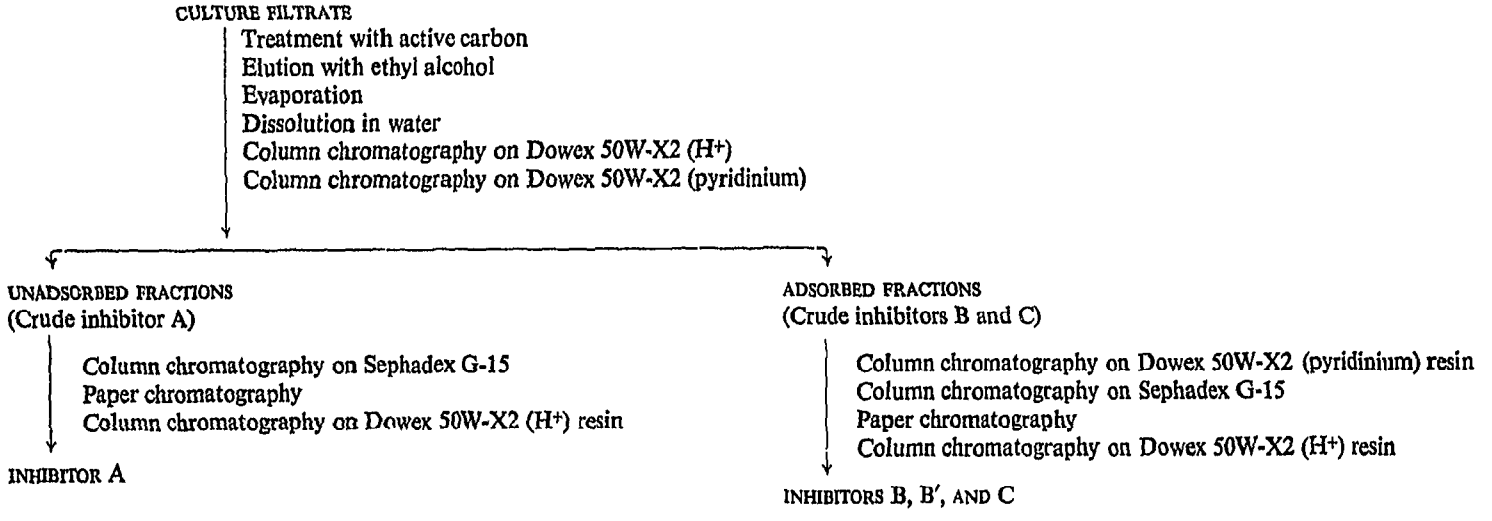
In the previous communication[5], we reported that the amylase inhibitor produced by *Streptomyces* sp. strongly inhibited animal alpha amylase [(1 → 4)-α-D-glucan glucanohydrolase, EC 3.2.1.1], bacterial saccharogenic alpha amylase [(1 → 4)-α-D-glucan glucanohydrolase, EC 3.2.1.1], and fungal glucoamylase [(1 → 4)-α-D-glucan glucohydrolase, EC 3.2.1.3], but not pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41), nor plant beta amylase [(1 → 4)-α-D-glucan maltohydrolase, EC 3.2.1.2].

Shainkin and Birk[6], in a study of the amylase inhibitor of wheat, separated two proteinaceous inhibitors by column chromatography on DEAE- and CM-cellulose. Similar findings were reported by Silano and co-workers[7]. We found[8] that the amylase inhibitor produced by *Streptomyces* sp. can be separated by chromatography into four fractions (amylase inhibitors A, B, B′, and C) and these contained

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

†Part III of the series: Studies on Amylase Inhibitors Produced by *Streptomyces* sp.; see also refs. 5 and 8.

Chart I. Purification procedures for amylase inhibitors.



both carbohydrates (mainly D-glucose) and amino acids; their molecular weights ranged from 1300 to 4000.

This communication describes further purification of these amylase inhibitors and their action on some carbohydrolases and phosphorylases [(1 → 4)-α-D-glucan: orthophosphate α-D-glucosyltransferase, EC 2.4.1.1].

RESULTS

Further purification of amylase inhibitors. — The principle of the purification procedures for the amylase inhibitors is based upon the communication previously reported[8]. The procedures used here differ at three points from those previously reported. The first one, as shown in Chart I, is that the culture filtrate itself is used in the adsorption on active carbon, in order to simplify the purification procedure. Secondly, the amylase inhibitors were purified by paper chromatography. This procedure was used only for amylase inhibitor A in the previous report[8], but the technique is applied here for purification of all of the amylase inhibitors (A, B, B′, and C). The third modification involves rechromatography of the amylase inhibitors on Dowex 50W-X2 (H^+) resin at the final step. After chromatography on Dowex 50W-X2, the amylase inhibitor is completely free from the trace of impurities (carbohydrate) originating from the paper used for chromatography.

The first elution-pattern of the crude inhibitor from the column of Dowex 50W-X2 (pyridinium) resin is shown in Fig. 1. The three observed peaks of inhibitory activity were designated as inhibitors A, B, and C in the order of their elution. Amylase inhibitors A, B, and C were each subjected to column chromatography on Sephadex G-15, as shown in Figs. 2, 3, and 4. As shown in Fig. 3, the amylase inhibitor B was separated into two peaks. The first peak was designated inhibitor B and the second one inhibitor B′. After gel filtration, each amylase inhibitor was purified by

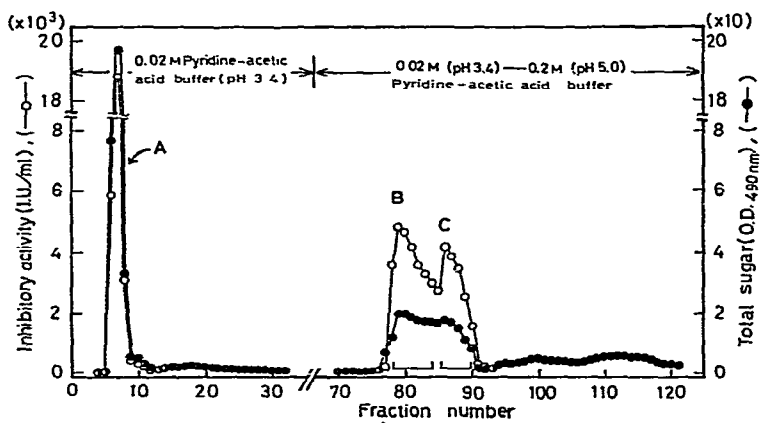


Fig. 1. Column chromatography on Dowex 50W-X2 (pyridinium) resin. Experimental details are given in the text. Fractions of peak I (inhibitor A), peak II (inhibitor B), and peak III (inhibitor C) were collected and subjected to further experiments.

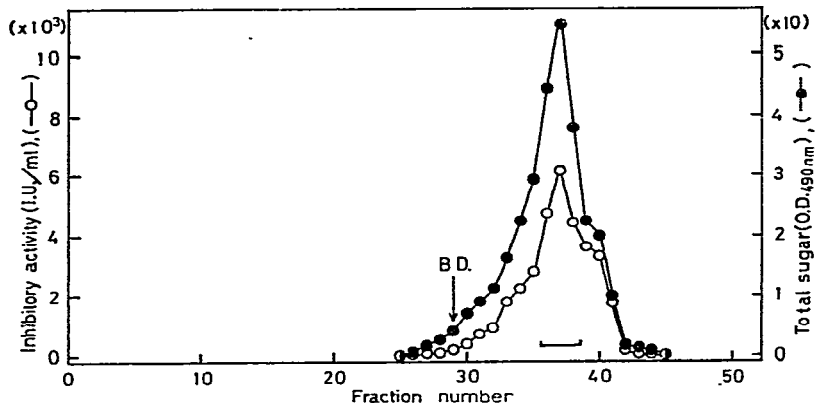


Fig. 2. Gel filtration of inhibitor A on Sephadex G-15. Experimental details are given in the text. The symbol B.D. designates the elution position of blue dextran.

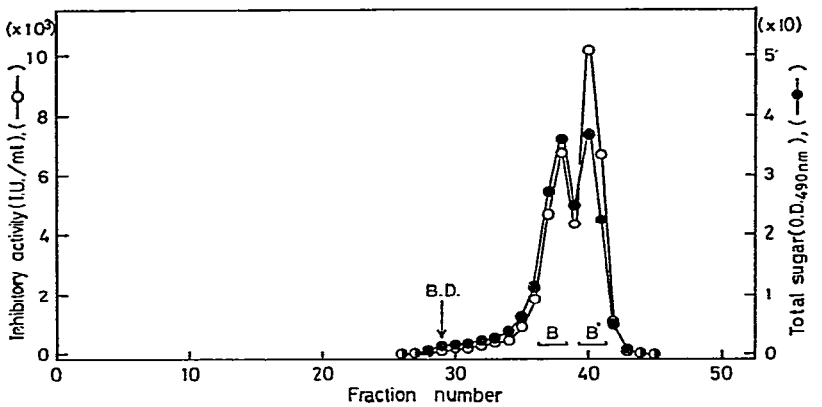


Fig. 3. Gel filtration of inhibitor B on Sephadex G-15. Experimental details are given in the test. Fractions of peak I (inhibitor B) and peak II (inhibitor B') were collected and subjected to further experiments.

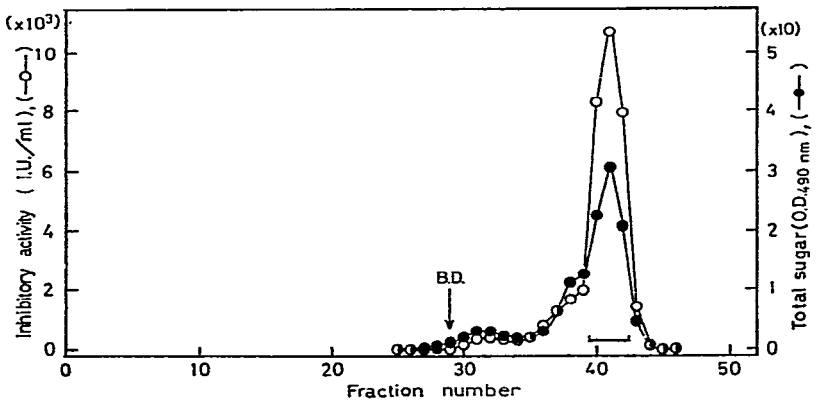


Fig. 4. Gel filtration of inhibitor C on Sephadex G-15. Experimental details are given in the text.

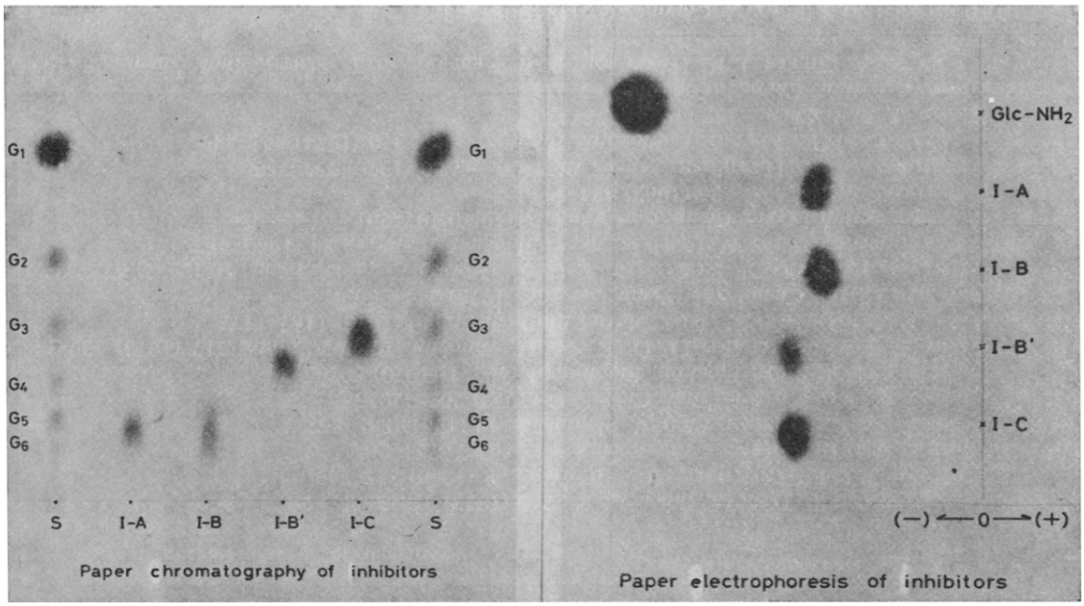


Fig. 5(left). Paper chromatography of inhibitors. Experimental details are given in the text. Paper-chromatographic analysis of each amylase inhibitor (~200 μg of total sugar as D-glucose) was performed. The symbols G_1, G_2, – G_6 denote D-glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose, respectively.

Fig. 6(right). Paper electrophoresis of inhibitors. Experimental details are given in the text. Glc-NH_2= 2-amino-2-deoxy-D-glucose (standard).

paper chromatography and by column chromatography on Dowex 50W-X2 (H^+) resin, each of the amylase inhibitors showed only one component by paper chromatography or paper electrophoresis, as shown in Figs. 5 and 6, although the amylase inhibitors A, B, B′, and C previously reported each had given two spots, either by paper chromatography or by paper electrophoresis[8]. The results of the series of purification steps are also summarized in Table I.

Action of crude amylase inhibitors on some carbohydrate hydrolases and phosphorylases. — As a preliminary experiment, the mixture of amylase inhibitors (B, B′, and C)* was tested against various carbohydrate hydrolases and phosphorylases. As shown in Table II, the amylase inhibitor inhibited potato and muscle phosphorylase, yeast and fungal α-D-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), and cycloamylose glucanotransferase [(1 → 4)-α-D-glucan 4-α-[(1 → 4)-α-glucano]transferase (cyclizing), EC 2.4.1.19]. However, the amylase inhibitors did not act on Q-enzyme [(1 → 4)-α-D-glucan:(1 → 4)-α-D-glucan 6-α-[(1 → 4)-α-glucano]transferase, EC 2.4.1.18], nor on dextransucrase [sucrose:(1 → 6)-α-D-glucan 6-α-glucosyltransferase, EC 2.4.1.5].

*The fraction adsorbed on Dowex 50W-X2 (pyridinium) column chromatography in Chart I.

TABLE I

PURIFICATION OF AMYLASE INHIBITORS

Steps	*Volume (ml)*	*Total activity (I.U.)*	*Specific activity (I.U./ mg sugar)*	*Recovery (%)*
		× 10^3	× 10^2	
1. Active carbon treatment	250*	785	0.30	100
2. Dowex 50W-X2 (H^+ resin)	300	760	6.0	97
3. Dowex 50W-X2 (pyridinium resin)				
Inhibitor A	30	167	7.4	21
Inhibitor B	70	259	19.4	33
Inhibitor C	70	184	21.9	23
4. Rechromatography				
Inhibitor B	90	145	20.4	19
Inhibitor C	120	129	24.7	16
5. Sephadex G-15				
Inhibitor A	11	58.0	11.2	7.4
Inhibitor B	11	43.4	14.0	5.5
Inhibitor B′	8.0	64.0	24.6	8.2
Inhibitor C	11	102	32.0	13
6. Paper chromatography				
Inhibitor A	1.0*	32.6	13.9	4.2
Inhibitor B	1.0*	30.7	13.7	3.9
Inhibitor B′	1.0*	45.6	25.3	5.8
Inhibitor C	1.2*	66.8	32.7	8.5
7. Dowex 50W-X2 (H^+ resin)				
Inhibitor A	8.0	30.9	14.4	3.9
Inhibitor B	6.0	29.5	14.8	3.8
Inhibitor B′	4.0	39.8	26.0	5.1
Inhibitor C	4.0	58.4	33.0	7.4

*Volume after concentration.

TABLE II

ACTION OF CRUDE AMYLASE INHIBITOR ON SOME CARBOHYDRATE HYDROLASES AND TWO KINDS OF PHOSPHORYLASE

Enzyme	*Substrate*	*pH*	*Inhibition*[a]
Muscle phosphorylase *a*	Starch	6.80	+
Potato phosphorylase	Starch	6.80	+
Mucor α-D-glucosidase	Starch	5.30	+
	Maltose	5.30	+
Yeast α-D-glucosidase	Maltose	6.80	+
Q-enzyme	Amylose	7.00	—
Dextransucrase	Sucrose	5.80	—
Cycloamylose glycosyltransferase	Amylose	7.00	+

[a]Each enzyme solution (0.5 ml, 10 units) was incubated with 0.5 ml of amylase inhibitor (1500 I.U.) for 10 min at 30° and the residual enzyme activity was assayed. Inhibitory ratings used were as follows: — (0 inhibition) or + (80–100% inhibition).

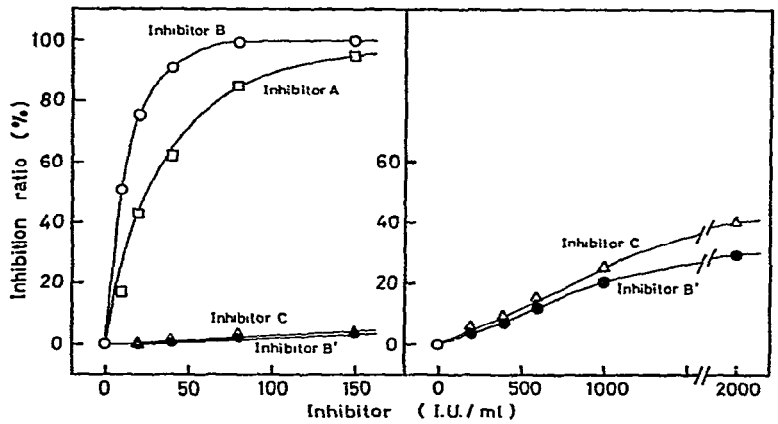


Fig. 7. Inhibition of muscle phosphorylase *a* by amylase inhibitors. The reaction mixture contained 2.5% of soluble starch (1.0 ml), amylase inhibitor (0.5 ml), 1.0 M phosphate buffer (pH 6.8, 0.5 ml), and phosphorylase (1.0 ml, 7.05 units).

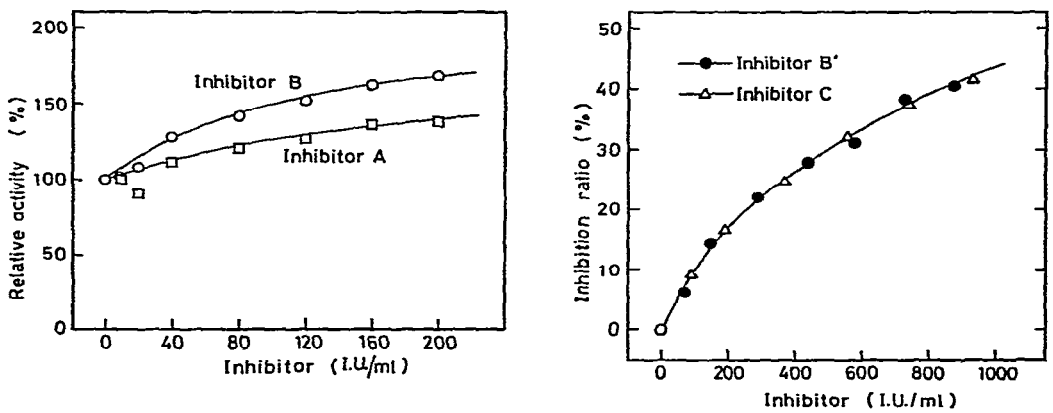


Fig. 8. Effect of inhibitor A and B on the α-D-glucosidase of yeast. The mixture contained 2.5% of maltose, amylase inhibitor (0.5 ml), 0.2M phosphate buffer (pH 6.8, 0.5 ml), and α-D-glucosidase (1.0 ml, 4.5 units).

Fig. 9. Inhibition of α-D-glucosidase by amylase inhibitors B' and C of yeast. The mixture contained 2.5% maltose (1.0 ml), amylase inhibitor (0.5 ml), 0.2M phosphate buffer (pH 6.8, 0.5 ml), and α-D-glucosidase (1.0 ml, 1.5 units).

Action of each amylase inhibitor on muscle phosphorylase a. — As shown in Fig. 7, the amylase inhibitors A and B strongly inhibited phosphorylase *a*, but B' and C showed little inhibition of phosphorylase *a*.

Action of each amylase inhibitor on yeast α-D-glucosidase. — As shown in Figs. 8 and 9, amylase inhibitors A and B enhanced the maltase activity of α-D-glucosidase, but amylase inhibitors B' and C inhibited this activity. The action of α-D-glucosidase on amylase inhibitors did not result in the liberation of D-glucose from amylase inhibitors which are glycopeptides[8], nor was the amylase inhibitor inactivated.

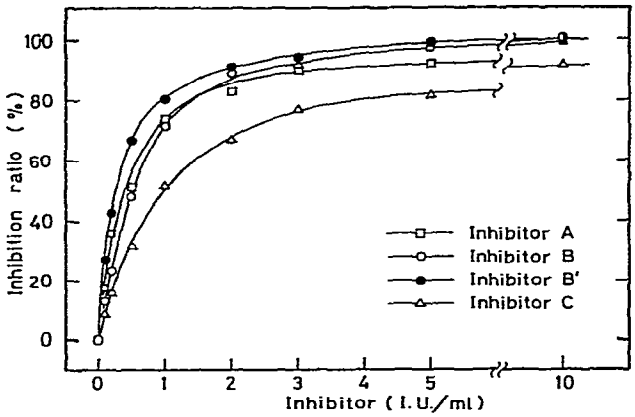


Fig. 10. Inhibition of cycloamylose glucanotransferase by amylase inhibitors. The mixture contained 0.05% of amylose dissolved in 0.1M phosphate buffer (pH 7.0, 0.3 ml), amylase inhibitor (0.1 ml), and cycloamylose transferase (0.1 ml, 4.7 units).

Action of each amylase inhibitor on cycloamylose glucanotransferase. — As shown in Fig. 10, each of the amylase inhibitors (A, B, B', and C) strongly inhibited the starch-splitting activity of cycloamylose glucanotransferase.

DISCUSSION

Inhibitors of plant amylase show only inhibition against alpha amylases, especially those of animal origin[9]. As previously reported by us[5], the amylase inhibitor of *Streptomyces* inhibits not only alpha amylase, but also glucoamylase. The four types of amylase inhibitor showed different behavior towards various carbohydrate hydrolases and phosphorylases. Amylase inhibitors A and B showed the same behavior, and B' and C also exhibited the same behavior; thus, A and B strongly inhibited phosphorylase *a* but activated yeast α-D-glucosidase. On the other hand, B' and C inhibited phosphorylase *a* very weakly, but substantially inhibited yeast α-D-glucosidase. Such an activation of glucosidase activity by an amylase inhibitor has never been shown before, as far as we are aware.

Although inhibitors A and B show similar behavior toward phosphorylase and yeast α-D-glucosidase, and migrate almost the same distance on paper chromatography and paper electrophoresis, they differ in properties by column chromatography on Dowex 50W-X2 (pyridinium) resin. In contrast, inhibitors B' and C display similar behavior toward phosphorylase and yeast α-D-glucosidase, and migrate to almost the same distance by paper chromatography and paper electrophoresis, but they show different properties by column chromatography on Dowex 50W-X2 (pyridinium).

As shown in the previous report[8], these inhibitors contain 5–7 residues of D-glucose and some molecules of amino acids. The properties of these peptides might determine the behavior of the inhibitors. It seems reasonable to conclude that the crude inhibitor is a mixture, as four components have been isolated and the components

shown to differ somewhat in their activity toward different carbohydrate hydrolases.

Further studies will be required in order to clarify whether the amylase inhibitors themselves also possess carbohydrate-hydrolase inhibitor activity or whether they are mixtures of various inhibitors. At present, it seems plausible to postulate that the amylase inhibitor occurs in multiple forms, and that each of the forms shows different behavior toward various kinds of carbohydrate hydrolases and phosphorylases.

EXPERIMENTAL

Microorganism. — The organism producing amylase inhibitors was isolated in our laboratory from soil, and was identified as a variety of *Streptomyces flavochromogenes*[10].

Analysis. — A. Assay of enzymes and amylase-inhibitor activities. (1) Assay of inhibitor activity. The assay system for the amylase inhibitor was as follows: A mixture consisting of 0.1 ml of 1% glucoamylase solution from *Rhizopus* sp. (commercial preparation, Amano Pharmaceutical Co.), test solution (0.2 ml), and water (0.7 ml) was incubated for 10 min at 40°* and then the residual amylase activity was measured by the following method. The mixture, consisting of 2.5 ml of 1% soluble starch solution, 0.5 ml of 0.2M acetate buffer (pH 5.5), and 0.5 ml of the treated amylase solution, was incubated at 40°. After 15 min, 1 ml of the mixture was withdrawn for determination of glucose by the micro-Bertrand method.

Inhibition activity is expressed as the percent of inhibition (i.r.) as follows:

$$\text{I.r. } (\%) = \frac{A - B}{A} \times 100,$$

where A is the activity in amylase units in the absence of inhibitor, and B is that in the presence of inhibitor. One unit of amylase (A.U.) is defined as the activity equivalent to 0.1 mg of D-glucose liberated per min, per ml of reaction mixture at 40°.

The following definition is used for the inhibitor unit: one unit of inhibitor (I.U.) is equivalent to a decrease of 1 A.U. under the foregoing conditions.

(2) Assay of α-D-glucosidase activity. α-D-Glucosidase activity was determined by measuring the amount of D-glucose liberated from the substrate. The mixture contained 1.0 ml of 2.5% maltose solution, 0.5 ml of 0.5M phosphate buffer (pH 5.2 for fungal enzyme, pH 6.8 for yeast enzyme), enzyme solution, and deionized water in a final volume of 3.0 ml. After incubation for 60 min at 30°, the reaction was stopped by heating the mixture for 5 min in a boiling-water bath. The amount of D-glucose formed was determined by the D-glucose oxidase method[11]. One unit of α-D-glucosidase activity is defined as the amount of enzyme that will produce 1 μmol of D-glucose from maltose under the foregoing conditions.

*Preincubation of amylase and the amylase inhibitor is required for the amylase inhibitors of higher plants[7], but it is not necessary for the microbial amylase-inhibitors.

(*3*) *Assay of phosphorylase activity.* Phosphorylase activity was determined by measuring the amount of inorganic phosphate liberated by heating for 7 min in 0.5M sulfuric acid; this corresponds to inorganic phosphate originating from α-D-glucosyl phosphate. The mixture contained 1.0 ml of 2.5% soluble-starch solution, 0.5 ml of 1.0M phosphate buffer (pH 6.8), enzyme solution, and deionized water in a final volume of 3.0 ml. After incubation for 60 min at 30°, the reaction was stopped by adding 9.0 ml of magnesia mixture. The amount of α-D-glucosyl phosphate produced was determined by the method of Nakamura[12]. One unit of phosphorylase activity is defined as the amount of enzyme that will produce 1 μmol of α-D-glucosyl phosphate under the foregoing conditions.

(*4*) *Assay of Q-enzyme activity.* Q-Enzyme activity was assayed by an iodine-staining method similar to that reported by Igaue[13]. The mixture consisted of 5 ml of 0.2% amylose in 0.5M potassium chloride (pH 4.2), 2 ml of 0.4M sodium citrate buffer (pH 7.0), enzyme solution, and deionized water to make to 10 ml. After incubation for 60 min at 25°, a 1-ml aliquot was pipetted into a 100-ml volumetric flask containing 5 ml of 0.01M iodine acidified with 1 ml of 0.5M hydrochloric acid, and the solution was made up to 100 ml with deionized water. One unit of Q-enzyme activity is defined as the amount of enzyme preparation that decreases the absorbance at 700 nm of the amylose–iodine complex by 1% per min in the standard reaction-mixture.

(*5*) *Assay of cycloamylose glucanotransferase activity.* Cycloamylose glucanotransferase activity was assayed as the dextrinizing activity, because the latter may be used, as Nakamura and Horikoshi have reported[14], as the expression of the cycloamylose glucanotransferase activity in the case of alpha amylase-free enzyme. The mixture, containing 0.1 ml of the enzyme, 0.1 ml of deionized water, and 0.3 ml of 0.05% amylose in 0.2M acetate buffer (pH 7.0), was incubated for 20 min at 40°. The reaction was stopped with 4 ml of 0.2M hydrochloric acid. To the mixture were added 0.5 ml of 0.01M iodine and deionized water to make a volume of 10 ml, and the absorbancy at 700 nm was measured. One unit of the enzyme is defined as the amount of enzyme that produces a 10% decrease in the intensity of blue color of the amylose–iodine complex under the foregoing conditions.

(*6*) *Assay of dextransucrase activity.* This activity was determined by a method similar to that reported by Kobayashi and Matsuda[15]. The mixture consisted of 2 ml of 2.5% of sucrose solution, 1 ml of enzyme solution, 0.9 ml of 0.2M citrate–hydrochloric acid (McIlvaine's buffer) buffer (pH 5.2), and 0.1 ml of deionized water. The mixture was incubated for 60 min at 30°. Afterwards, the reaction was stopped by heating the mixture for 5 min in a boiling-water bath. The amount of D-fructose formed was determined by the Nelson–Somogyi method[16,17]. One unit of dextransucrase is defined as the amount of enzyme that forms 1 μmol of D-fructose from sucrose under the foregoing conditions.

B. Determination of total sugar. The phenol–sulfuric acid method[18] with D-glucose as the standard was used to determine the total sugar content of the inhibitor. The absorbance was measured at 490 nm.

Enzyme preparations. — Purified potato phosphorylase and crystalline α-D-glucosidase of *Mucor javanicus* were gifts from Prof. Toshio Fukui of Osaka University and Prof. Yukio Suzuki of Okayama University, respectively. Rabbit-muscle phosphorylase *a* and yeast α-D-glucosidase were purchased from Boehringer Mannheim GmbH and Sigma Chemical Co., respectively. A partially purified potato Q-enzyme was prepared as described by Bourne and Peat[19]. A partially purified, extracellular dextransucrase was prepared from *Leuconostoc mesenteroides* NRRL B-512 according to the method of Tsuchiya *et al.*[20,21]. A partially purified, cycloamylose glucanotransferase preparation was donated by Dr. Nobuyuki Nakamura of Nihon Shokuhin Kako Co.

Chemicals. — Amylose (mol. wt. ~ 2100) was purchased from Nakarai Chemical Ltd.

Procedures for purification of amylase inhibitors. — A. Purification of amylase inhibitors by column chromatography on Dowex 50W-X2 (H^+) resin. Inhibitor solution (250 ml, 26 g as D-glucose) after treatment with active carbon was chromatographed on a column (2.5 × 60 cm) of Dowex 50W-X2 (H^+). The column was developed with about 2000 ml of deionized water and then with 3000 ml of 0.4M pyridine–acetic acid buffer (pH 4.6). The elution flow-rate was 40 ml per h and the effluent was collected in 20-ml fractions.

B. Purification of amylase inhibitors by column chromatography on Dowex 50W-X2 (pyridinium). Crude inhibitor (10 ml, 1265 mg as D-glucose) contained in 0.01M hydrochloric acid was applied to a column (1.9 × 40 cm) equilibrated with 0.02M pyridine–acetic acid buffer (pH 3.4). The column was developed with about 500 ml of the same buffer and then with a linear gradient utilizing 350 ml of the same buffer and 350 ml of 0.2M pyridine–acetic acid buffer (pH 5.0). The elution flow-rate was 30 ml per h and the effluent was collected in 10-ml fractions.

C. Purification of amylase inhibitor by column chromatography on Sephadex G-15. Inhibitor solution (1 ml, ~ 200 mg of inhibitor A, 70 mg of inhibitor B, and 50 mg of inhibitor C as D-glucose, respectively) was applied to a column (1.6 × 136 cm) of Sephadex G-15 equilibrated with 0.1M acetic acid. The elution was performed with the same solution at a flow-rate of 10 ml per h and 3.8-ml fractions were collected.

D. Purification of amylase inhibitor by paper chromatography. Paper chromatography was performed with Toyo filter paper No. 50 with 12:8:5 butyl alcohol–pyridine–water. Inhibitor solutions containing ~ 10 mg of sugar (as D-glucose) per sheet of paper (40 × 40 cm) were streaked 5 cm from the bottom of the paper. Triple-ascending chromatography of inhibitors A and B, and double-ascending chromatography of inhibitors B′ and C was performed. Each active part of the paper chromatogram was excised and extracted with deionized water. By this extraction, a trace of impurity (saccharides) that originated from the filter paper was eluted, together with the amylase inhibitor. Column chromatography on Dowex 50W-X2 (H^+) was used to remove the impurity. The column (1.0 × 15 cm) was developed with deionized water and then with 0.5M pyridine. Fractions (2 ml) of the effluent were collected at a

flow-rate of 4 ml per h. The inhibitors were detected and identified by the silver nitrate reagent.

E. Paper electrophoresis of amylase inhibitors. Paper-electrophoretic analysis of amylase inhibitors (~ 200 μg, total sugar as D-glucose) was performed at ~ 12 V/cm for 4 h with Toyo filter paper No. 50 and the following buffer: 80% formic acid–acetic acid–water (5:15:80, pH 1.9), with 2-amino-2-deoxy-D-glucose as standard; detection was with silver nitrate.

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