Amylase Inhibitors Produced by *Streptomyces* sp. No. 280

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Fourteen kinds of amylase inhibitor (AI) were obtained by active carbon column chromatography and other procedures. The molecular weights of the AIs were estimated to be 310~1300 by gel filtration. They seem to have 1~4 glucose residues and one nitrogen per molecule. All the AIs have almost the same inhibitory activity against glucoamylase. High molecular weight AIs have much stronger activity toward α -amylase and phosphorylase *a* than low molecular weight AIs, but the latter have much stronger inhibitory activity toward sucrase and α -glucosidase than the former. Four kinds of AI were hydrolyzed by β -amylase which changed their inhibitory activities.

Schmidt *et al.*¹⁾ reported that amylase inhibitor BAYe 4609 was hydrolyzed by β -amylase, α -amylase and mineral acid. Recently, Ueda and Marshall,²⁾ and Hidaka *et al.*³⁾ reported that the inhibitory activities of BAYe 4609 and BAYg 5421 toward α -amylase, glucoamylase, sucrase and α -glucosidase were changed after modification by some kinds of amylase.

In previous papers,^{4,5)} amylase inhibitors (AI) produced by *Streptomyces* sp. No. 280 were reported to consist of five kinds (A₁, A₂, B, B' and C). After being hydrolyzed by α -amylase, A₁ and A₂ decreased in inhibitory activity toward α -amylase but increased in inhibitory activity toward sucrase. Only A₁ was hydrolyzed by β -amylase with a change in inhibitory activity.

In this study, in order to further investigate the properties of AIs produced by *Streptomyces* sp. No. 280, we purified fourteen AIs and studied their inhibitory activities, modification by β -amylase and physicochemical properties.

MATERIALS AND METHODS

Microorganism. The organism used was identified as *Streptomyces flavochromogenes*,⁶⁾ it had been isolated in our laboratory from soil.

Cultivation. Flask cultivation for production of AIs was carried out at 30°C for 6 days in 500 ml of 3% oatmeal medium on a rotary shaker. The pH was not adjusted.

Enzymes. Hog pancreatic α -amylase, sweet potato β -amylase, rabbit muscle phosphorylase *a* and yeast α -glucosidase were purchased from Boehringer Mannheim GmbH. Porcine small intestinal sucrase was prepared by freeze-thaw treatment according to the method of Marshall *et al.*⁷⁾ Glucoamylase from *Rhizopus* sp. was a commercial preparation from Amano Pharmaceutical Co.

Assay of inhibitory activities toward some carbohydrases and phosphorylase *a*. Assays of inhibitory activities toward glucoamylase,⁸⁾ pancreatic α -amylase,⁹⁾ muscle phosphorylase *a*,⁴⁾ small intestinal sucrase⁹⁾ and yeast α -glucosidase⁴⁾ were done according to the methods reported previously. One unit of inhibitory activity for each enzyme was defined as the decrease of one unit of each enzyme activity.

Active carbon column chromatography. In this study, we used active carbon column chromatography for purification of AIs. Active carbon for column chromatography (Nakarai Chemicals Ltd.) was agitated in 2N HCl for 2 hr and washed with water. The active carbon was mixed with the same amount of Celite 545 (Nakarai Chemicals Ltd.) and packed into a column (4.5 × 40 cm) equilibrated with water. An AI was applied on this column and washed with water, and then stepwise elution was done with 0~60% ethanol.

Paper chromatography and paper electrophoresis. Paper chromatography and paper electrophoresis of AIs were carried out as described in previous papers.^{5,9)}

Estimation of molecular weight of AIs. A sample solution

was applied on a column of Sephadex G-15 (2.4 × 140 cm) equilibrated with 0.1 M acetic acid. Elution was performed with the same acid solution at a flow rate of 12 ml/hr, and 2.0 ml fraction were collected. Maltooligosaccharides used as standards were prepared from maltodextrin (Hayashibara Biochemical Institute) by paper chromatography. The void volume of the column was measured with blue dextran.

High performance liquid chromatography (HPLC) of AIs. HPLC of AIs was carried out with a Hitachi 638-30 HPLC using a column (4.6 × 250 mm) packed with Zorbax NH₂ (Dupont). Elution was carried out with acetonitrile-water (70:30) at a flow rate of 1.0 ml/min, and amylase inhibitors were detected with a Mass detector (Applied Chromatography System Ltd.).

Determination of glucose and total sugar. The glucose oxidase method¹⁰⁾ and phenol-sulfuric acid method¹¹⁾ were used to determine glucose and total sugar, respectively.

Determination of reducing power of AIs. The Somogyi-Nelson method^{12,13)} was used to determine the reducing power of AIs with glucose as standard.

Determination of the reducing end of AIs. The reducing end of an AI was determined as follows: After reduction of the AI with NaBH₄, the AI was hydrolyzed with 2 N HCl at 100°C for 2 hr, and Cl⁻ was removed with Amberlite IR-45 (OH⁻). The AI hydrolyzate was analyzed as trimethylsilyl (TMS) derivatives by gas-liquid chromatography (GLC). GLC was carried out with a Nihondenshi GLC Model JGC-20 KFP.

RESULTS

Preparation of AIs and hydrolyzed AIs

1) **Summary of purification.** In order to get small molecule AIs, 6 days cultivation was done and followed by purification. Some of them were purified after hydrolysis of the crude AI with 2 N trifluoroacetic acid (TFA) at

TABLE I. PURIFICATION PROCEDURES FOR AMYLASE INHIBITORS

TABLE II. PURIFICATION PROCEDURES FOR HYDROLYZED AMYLASE INHIBITORS

Culture filtrate	
Adsorption on active carbon	
Elution with ethyl alcohol	
Evaporation to dryness	
Dissolved in water	
Column chromatography on Dowex 50W (H ⁺)	
Hydrolysis of crude inhibitor with 2 N TFA at 100°C for 2 hr	
Column chromatography on Dowex 50W (H ⁺)	
Column chromatography on active carbon	
	F ₁ F ₂ F ₄ F ₃
Paper chromatography	
Column chromatography on Dowex 50W (H ⁺)	
Gel filtration on Sephadex G-10	
	AJ-H ₁ H ₂ H ₃

100°C for 2 hr. Purification of an AI could be done efficiently by active carbon column chromatography in addition to the methods described in a previous paper.⁴⁾ The purification steps are shown in Tables I and II. The results of purification are summarized in Tables III and IV.

AI-A', A₁, A₂, B₁, B₂, B₃, B₄, B₅, C, D and E were separated from native AI, and AI-H₁, H₂ and H₃ were separated from hydrolyzed AI.

2) *Paper chromatography of purified AIs.* The results of paper chromatography of purified AIs are shown in Fig. 1. Each AI had a different *R_f* value and gave a single spot.

3) *Paper electrophoresis of purified AIs.* On paper electrophoresis also, each AI gave a single spot and had a different *R_m*, and a small molecular AI moved to the cathode much faster than a larger molecular AI.

4) *HPLC of purified AIs.* The retention times on HPLC of purified AIs are shown in Table V. Each AI gave a single peak and some of them showed the same retention time.

Molecular weights of AIs

The molecular weights of AIs were estimated by gel filtration. The results are shown in Table VI. The molecular weights of AIs were distributed from 310 to 1300.

Ratio of total sugar content to reducing sugar content of AIs

The ratios are shown in Table VI.

Hydrolysis of AIs with 2 N TFA

AI-A', A₁, B₁, B₂, B₃, B₄ and B₅ were hydrolyzed with 2 N TFA at 100°C for 2 hr. The increase of reducing sugar in the reaction mixture stopped within 2 hr. The glucose content of the reaction mixture was determined by the glucose oxidase method. The increase of reducing sugar content agreed with the glucose content of the reaction mixture in each AI, so these AIs released only glucose on hydrolysis with TFA.

TABLE III. SUMMARY OF PURIFICATION PROCEDURES FOR AMYLASE INHIBITORS

	Total sugar (mg)	SA* (IU**/mg)	Total activity (IU $\times 10^{-3}$)	Yield (%)
Crude AI	61600	148	9100	100
Dowex 50W (H ⁺)	12000	723	8680	95.3
Carbon column				
F'	3440	933	3210	35.3
F''	2700	894	2414	26.5
F''' → ***	557	584	296	3.2
Dowex 50W (pyridine)				
F ₀ '''	37.0	55.7	2.1	trace
F ₁ ''	924	1340	1240	13.6
F ₂ ''	459	1136	521	5.7
F ₃ ''	557	808	450	4.9
Amberlite (pyridine)				
F ₁ '	82.6	402	33	0.4
F ₂ '	653	515	336	3.7
F ₃ '''	275	820	225	2.5
F ₄ '	213	1630	347	3.8
Paper chromatography				
F ₁ ' AI-A'	33.0	603	19.9	0.2
F ₂ ' AI-A ₁	93.7	500	46.9	0.5
AI-A ₂	124	641	79.5	0.9
F ₄ ' AI-C	33.2	1820	60.4	0.7
AI-D	29.5	1990	58.7	0.6
AI-E	10.5	1820	19.1	0.2
F ₁ '' AI-B ₁	621	751	466	5.1
F ₂ '' AI-B ₃	135	1030	139	1.5
AI-B ₅	54	1720	93.2	1.0
F ₃ '' AI-B ₂	211	820	173	1.9
AI-B ₄	31.0	1120	34.7	0.4
Dowex 50W (H ⁺)				
Sephadex G-15				
AI-A'	10.5	856	9.0	0.1
AI-A ₁	50.2	517	25.9	0.3
AI-A ₂	98.5	701	69.0	0.8
AI-B ₁	339	899	304	3.4
AI-B ₂	208	916	191	2.1
AI-B ₃	50.9	1140	58.0	0.6
AI-B ₄	23.0	1180	27.1	0.3
AI-B ₅	32.7	1990	65.0	0.7
AI-C	10.6	1960	20.7	0.2
AI-D	15.2	2270	34.5	0.4
AI-E	5.6	1930	10.8	0.1

* SP, specific activity.

** IU, inhibitory activity units toward glucoamylase.

*** Further purification was not successful for these fractions.

Elemental analysis of AIs

The results of elemental analysis of AIs are shown in Table VI. From these results, it was considered that each AI has one nitrogen per molecule, so we estimated the molecular formula. The smallest AIs (D, H₁, H₂ and H₃) have nineteen carbon atoms per molecule.

Inhibitory activity of AIs toward some carbohydrate hydrolases and phosphorylase a

In a previous paper, we compared the inhibitory activity of each AI as specific activity (IU/mg as glucose).⁵⁾ So, in this study, we compared the activity as the inhibitory units per mmol of AI. The results of inhibitory

TABLE IV. SUMMARY OF PURIFICATION PROCEDURES FOR HYDROLYZED AMYLASE INHIBITORS

	Total sugar (mg)	SA* (IU**/mg)	Total activity (IU $\times 10^{-3}$)	Yield (%)
Crude AI	2807	712	1990	100
Hydrolysis of AI				
Dowex 50W (H ⁺)	820	557	457	22.9
Carbon column				
F ₁	39.8	422	17.6	0.9
F ₂	64.9	1530	99.4	5.0
F ₃ ***	81.0	632	51.2	2.6
F ₄	97.9	1280	125	6.3
Paper chromatography				
F ₁ AI-H ₁	33.0	795	26.2	1.3
F ₂ AI-H ₂	50.5	1799	111	5.6
F ₄ AI-H ₃	46.3	2230	103	5.2
Dowex 50W (H ⁺)				
AI-H ₁	31.2	804	21.5	1.3
AI-H ₂	45.9	2530	121	6.0
AI-H ₃	44.0	2150	94.6	4.7
Sephadex G-10				
AI-H ₁	30.0	1010	30.3	1.5
AI-H ₂	39.9	2920	116	5.8
AI-H ₃	32.8	2830	92.8	6.6

* SA, specific activity.

** IU, inhibitory activity units.

*** Further purification was not successful for this fraction.

FIG. 1. Paper Chromatogram of Amylase Inhibitors.

S, maltooligosaccharides used as standards.

activity of AIs are shown in Table VII. All AIs have almost the same inhibitory activity toward glucoamylase. High molecular AIs inhibited α -amylase much stronger than low molecular AIs and only high molecular AIs

(AI-A', A₁ and A₂) inhibited phosphorylase α , A₁ especially has a strong inhibitory activity. A moderate molecular AI (AI-B₅) has a very strong inhibitory activity toward sucrase. High molecular AIs did not inhibit yeast α -gluco-

sidase, but low molecular AIs (AI-C, D, H₁, H₂ and H₃) inhibited the enzyme, hydrolyzed AIs especially (AI-H₁, H₂ and H₃) had a

strong inhibitory activity.

TABLE V. HPLC OF PURIFIED AIs

Chromatographic conditions: column, Zorbax™ NH₂ (4.6 × 250 mm); eluent, CH₃CN-H₂O = 70:30; flow rate, 1 ml/min.

Standard	Retention time	AI	Retention time
G ₁	1.00	AI-H ₃	1.49
G ₂	1.39	AI-H ₁	1.89
G ₃	1.92	AI-E	1.49
G ₄	2.71	AI-D	1.61
G ₅	3.81	AI-C	1.89
G ₆	5.23	AI-B ₅	2.20
		AI-B ₄	2.76
		AI-B ₃	3.07
		AI-B ₂	3.81
		AI-B ₁	4.22
		AI-A ₂	4.22
		AI-A ₁	4.96

* As standards, a maltose series was used.

TABLE VI. ELEMENTAL ANALYSIS OF PURIFIED AMYLASE INHIBITORS AND HYDROLYZED AMYLASE INHIBITORS

	Elemental analysis	Molecular formula	TS/RS*	M.W. ^a	M.W. ^b
AI-A ₁	C: 42.28 N: 1.30 H: 6.48	C ₃₈ H ₆₉ NO ₃₄	6.62	1083	1300
AI-A ₂	C: 43.33 N: 1.37 H: 6.46	C ₃₇ H ₆₅ NO ₃₁	5.98	1019	1100
AI-B ₁	C: 42.94 N: 1.38 H: 6.56	C ₃₆ H ₆₆ NO ₃₁	5.45	1008	1050
AI-B ₂	C: 44.04 N: 1.35 H: 6.68	C ₃₆ H ₆₉ NO ₃₁	4.91	1011	900
AI-B ₃	C: 43.26 N: 1.61 H: 6.49	C ₃₁ H ₅₆ NO ₂₆	4.17	858	800
AI-B ₄	C: 43.75 N: 1.60 H: 6.69	C ₃₂ H ₅₈ NO ₂₆	3.39	872	600
AI-B ₅	C: 43.37 N: 1.94 H: 6.76	C ₂₆ H ₄₈ NO ₂₁	2.95	710	600
AI-D	C: 43.18 N: 2.66 H: 6.68	C ₁₉ H ₃₅ NO ₁₆	1.97	533	310
AI-H ₁	C: 41.72 N: 2.51 H: 7.03	C ₁₉ H ₃₉ NO ₁₇	1.64	553	580
AI-H ₂	C: 45.38 N: 2.76 H: 7.07	C ₁₉ H ₃₆ NO ₁₄	2.07	502	550
AI-H ₃	C: 43.96 N: 2.65 H: 7.15	C ₁₉ H ₃₃ NO ₁₃	2.04	483	500

* TS/RS, ratio of total sugar content to reducing sugar content.

^a M.W. was calculated from molecular formula.

^b M.W. was estimated by gel filtration on a Sephadex G-15 column.

Modification of AIs by β -amylase

In our previous work,⁵⁾ only A₁ was hydrolyzed by β -amylase. As we could obtain many newly purified AIs this time, we tried modification of these AIs by β -amylase. AI-A₁, B₁, B₂ and B₄ were hydrolyzed by β -amylase, but other AIs (AI-A₂, B₃, B₅ and D) were not. The results are shown in Table VIII. AI-A₁, B₁, B₂ and B₄ released one mol of maltose and their inhibitory activities changed toward glucoamylase, α -amylase and sucrase, as shown in Table VIII. From these results, it was assumed that these AIs have more than two glucose units at the nonreducing end.

Determination of reducing end of AIs

Determination of the reducing end of AIs (AI-A₁, A₂, B₁, B₂, B₃, B₄, B₅ and D) was tried. The TMS derivatives of hydrolyzates of re-

TABLE VII. INHIBITORY ACTIVITY OF PURIFIED INHIBITORS*

Enzyme	AI-A'	AI-A ₁	AI-A ₂	AI-B ₁	AI-B ₂	AI-B ₃	AI-B ₄
Glucoamylase (<i>Rhizopus</i> sp.)	38.2	19.0	23.3	27.2	25.0	26.4	22.2
α -Amylase (Hog pancreas)	39.6	42.3	13.3	2.31	1.73	0.93	5.78
Sucrase (Hog intestine)	0.07	0.01	0.03	0.11	0.25	0.22	0.05
Phosphorylase <i>a</i> (Rabbit muscle)	5.81	140.6	1.68	0	0	0	0
α -Glucosidase (Yeast)	0	0	0	0	0	0	0

Enzyme	AI-B ₅	AI-C	AI-D	AI-E	AI-H ₁	AI-H ₂	AI-H ₃
Glucoamylase (<i>Rhizopus</i> sp.)	32.6	25.6	24.9	21.7	9.22	33.6	32.1
α -Amylase (Hog pancreas)	0.44	0.83	0.47	1.07	0.35	0.20	0.81
Sucrase (Hog intestine)	0.69	0.11	0.27	0.05	trace	0.06	0.01
Phosphorylase <i>a</i> (Rabbit muscle)	0	0	0	0	0	0	0
α -Glucosidase (Yeast)	0	0.13	0.06	0.13	0.38	1.46	2.37

* These figures are inhibitory activity (IU/m mol).

TABLE VIII. MODIFICATION OF AMYLASE INHIBITORS BY β -AMYLASE

AI	Increase of reducing sugar (fold)	Residual inhibitory activity (%)		
		Glucoamylase	α -Amylase	Sucrase
AI-A ₁	1.86	100	5.20	100
AI-B ₁	2.00	43.5	46.9	93.0
AI-B ₂	2.19	21.9	46.9	510
AI-B ₄	2.01	100	28.6	46.0

duced AIs gave peaks of α -D-glucose, sorbitol and β -D-glucose (AI-A₁, A₂, B₁, B₂, B₃, B₄ and B₅) or sorbitol only (AI-D). It became apparent that these AIs have one glucose unit at the reducing end.

DISCUSSION

Fourteen purified AIs were obtained by active carbon column chromatography, Dowex 50W column chromatography, paper chromatography, etc. Each of these AIs gave a single spot or single peak on paper chromatography,

paper electrophoresis and HPLC. These AIs have different inhibitory activities toward hog pancreatic α -amylase, hog small intestine sucrase, rabbit muscle phosphorylase *a* and yeast α -glucosidase. However, all the AIs have almost the same inhibitory activity toward glucoamylase (19~38 IU/mmol). High molecular AIs inhibited α -amylase much stronger than low molecular AIs, and after modification of AIs with amylase or organic acid, the inhibitory activity toward α -amylase decreased. Such results were seen for every AI. Schmidt *et al.*¹⁾ reported that the inhibitory activity to-

ward sucrase was increased after hydrolysis with mineral acid. In this study, a moderate molecular AI (AI-B₅) was shown to have the strongest inhibitory activity toward sucrase (0.69 IU/mmol) but AI-B₂ modified by β -amylase had 5 times higher inhibitory activity toward sucrase than that of original AI-B₂ (modified AI-B₂, 1.28 IU/mmol; original AI-B₂, 0.25 IU/mmol). (Table VIII and IX). AI-A', A₁ and A₂ inhibited phosphorylase *a*, and then after modification of them with amylase or mineral acid, the inhibitory activity toward phosphorylase *a* was lost. AI-A', A₁, A₂, B₁, B₂, B₃, B₄ and B₅ did not inhibit yeast α -glucosidase, but AI-C, D, E, H₁, H₂ and H₃ inhibited this enzyme.

We thought that the inhibitors produced by *Streptomyces* sp. No. 280 were kinds of glycopeptides in the early study, but now we can't confirm the presence of glycopeptide-inhibitors. This may be because purification of the inhibitors was insufficient. However, there is also another possibility, that is, the nature of this strain changed and the strain became unable to produce the glycopeptide-inhibitors. Because in the early study⁸⁾ we didn't find inhibitors which were hydrolyzed by β -amylase, but now some inhibitors were found to be sensitive to β -amylase. In the previous studies,^{4,5)} only A₁ was hydrolyzed by β -amylase, but in this study, AI-A₁, B₁, B₂ and B₄ were hydrolyzed by this enzyme. On the other hand, A₂, B, B' and C in the previous studies were not hydrolyzed by β -amylase as AI-A₂, B₃, B₅ and D were not in this study.

We think that the structure of the inhibitors seems to be similar to that of reported oligosaccharide-inhibitors,^{2,14,15)} that is, they consist mainly of glucose with a mixture of an N-compound which has 13 carbon atoms and a glucose. Because the compounds obtained on hydrolysis of the inhibitors with TFA had 19 carbon atoms, and the formula of AI-H₃ was C₁₉H₃₃NO₁₃. The differences of the fourteen purified AIs may be caused by their different glucose chain lengths. However, some inhibitors produced by *Streptomyces* sp. No. 280 have properties different from the reported

oligosaccharide-inhibitors. That is to say, AI-A₂, B₃, B₄ and D aren't hydrolyzed by β -amylase, and AI-A', A₁ and A₂ inhibit phosphorylase *a*. From these results, it is suggested that these inhibitors have different structures from the reported oligosaccharide-inhibitors.

We don't know why in AI-A₂, for instance, the number of glucoses estimated from the ratio of total sugar content to reducing sugar content in Table VI seems to be 6, but that from the molecular formula seems to be 4 if the N-compound has 13 carbon atoms. The total sugar content value obtained by the phenol-sulfuric acid method for these inhibitors may be larger than the actual sugar content.

From these results, it is suggested that the inhibitory activity of AIs toward these carbohydrases and phosphorylase *a* is closely related to the glucose chain structure of each AI. Therefore, it is necessary to reveal the detailed relationship between the inhibitory activity and the glucose chain structure of an AI. More detailed investigation of the structure of AIs will be reported elsewhere.

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