Purification of α -Maltotetraose-forming *exo*-Amylase of *Pseudomonas stutzeri*—Two-forms of the Amylase and Their Enzymatic Properties[†]

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Two active forms (F-1 and F-2) of a maltotetraose-forming amylase [EC 3.2.1.60 *exomaltotetraohydrolase*] from *Pseudomonas stutzeri* were completely separated and highly purified by using Polybuffer exchanger PBETM 94, and their enzymatic properties were compared. The purified F-1 and F-2 both showed a single band in polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. The optimum pH for the action of F-1 and F-2 on starch was exactly the same at 8.0. The isoelectric points of F-1 and F-2 were estimated to be 5.6 and 5.3 by polyacrylamide gel electrofocusing. Except the properties above, other enzymatic properties were very similar for F-1 and F-2: 1) the optimum temperature (45°C at pH 8.0) and the molecular weight (5.5×10^4), 2) the stabilization by Ca^{2+} , and the inhibition by some metal ions and *N*-bromosuccinimide, 3) the inhibition by cyclomaltodextrins and the inability to hydrolyze cyclomaltodextrins, 4) the kinetic constants (K_m and k_o) for starch, glycogens and short chain amylose (DP=17), and 5) the type of inhibition and inhibitor constants (K_i) of some microbial α -amylase inhibitors. Both purified F-1 and F-2 attacked starch from the non-reducing end to produce α -anomer oligosaccharides; these results confirmed that both enzymes were *exo*- α -amylases as reported previously.

In the previous papers^{1,2)} we reported that a maltotetraose-forming amylase [EC 3.2.1.60 *exomaltotetraohydrolase*] (abbreviated as G_4 -amylase) from *Pseudomonas stutzeri* should be classified as an *exo*- α -amylase, because of its hydrolyzing starch from the non-reducing end to produce α -anomeric oligosaccharides. The partially purified enzyme, eluted from a Sephadex G-100 column, contained F-1 and F-2 forms of G_4 -amylase and showed an optimum at pH 8.0~8.5, its pH activity curve having a shoulder near pH 6.0, while the purified F-2 showed an optimum at pH 6.0~6.5.^{1,2)} Robyt and Ackerman³⁾ reported the optimum pH of their enzyme to be 8.0, its pH activity curve also having a shoulder near

pH 6.0. In order to elucidate this discrepancy of optimum pH before and after the separation of these forms, it is necessary for the two forms of this G_4 -amylase to be separated completely and purified to homogeneity.

In this report the further separation and purification of the two forms from partially purified G_4 -amylase preparation were performed to compare the enzymatic properties of F-1 with those of F-2. For this purpose, F-1 and F-2 were separated and highly purified by using a novel chromatography method, a chromatofocusing method,^{4,5)} instead of the DEAE-cellulose chromatography used in the previous studies.^{1,2)} Purified F-1 and F-2 showed a single band in PAGE* and

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* Abbreviations: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PMSF, phenylmethanesulfonyl fluoride; NBS, *N*-bromosuccinimide; IAA, monoiodoacetic acid; EFA, ethoxyformic anhydride; PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid; α -CD, cyclomaltohexaose; β -CD, cyclomaltoheptaose; γ -CD, cyclomaltooctaose; PAGE, polyacrylamide gel electrophoresis in the absence of SDS; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS.

SDS-PAGE. Except the isoelectric points and the mobility in PAGE of F-1 and F-2, the properties of these forms compared in this study were very similar as described below.

MATERIALS AND METHODS

Materials. Soluble starch, shellfish glycogen, oyster glycogen and short chain amylose (DP = 17) were the same commercial products described previously.^{1,2)} α -CD, β -CD and γ -CD were gifts from Nihon Shokuhin Kako Co., Ltd. Non-labeled and reducing end-labeled (¹⁴C)-malto-oligosaccharides were prepared as described previously.^{1,2)} Sephadex G-100, Polybuffer™ 96 and Polybuffer exchanger PBE™ 94 were purchased from Pharmacia Fine Chemicals. Microbial α -amylase inhibitors used were gifts from Professor S. Murao, University of Osaka Prefecture, Professor S. Ueda, Kyushu University, Taisho Pharmaceutical Co., Ltd., and Meiji Seika Co., Ltd. as described previously.^{1,2)} Other reagents were commercially obtained and of the highest grade obtainable.

Analyses. The protein content was measured by the method of Lowry *et al.*,⁶⁾ using bovine serum albumin as a standard. Reducing sugar and total sugar were determined by the Nelson-Somogyi method⁷⁾ and the phenol-sulfuric acid method,⁸⁾ respectively, and calculated as maltotetraose or glucose equivalents. The products of enzymatic hydrolysis of starch or glycogen were identified by paper chromatography as described previously.^{1,2)} Two dimensional paper chromatography was performed under the same conditions as reported previously,^{1,2)} in order to examine the action of F-1 and F-2 on non-labeled or reducing end-labeled maltooligosaccharides.

Assay of amylase activity. Fifty μ l of enzyme solution was incubated with 200 μ l of 0.5% soluble starch buffered with 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM CaCl₂ at 30°C for 5 min. Instead of directly adding 250 μ l of Somogyi reagent to the reaction mixture, the enzyme reaction was stopped by adding 50 μ l of 1 N acetic acid. The enzyme was denatured by heating for 5 min in a boiling bath. Reducing sugar in the reaction mixture was determined as described above. One unit of the amylase was the amount of the enzyme which catalyzed the hydrolysis of 1 μ mol of glycosidic bonds per min.

Polyacrylamide gel electrophoreses. PAGE and SDS-PAGE were carried out by the methods of Davis⁹⁾ and Weber and Osborn,¹⁰⁾ respectively. Electrofocusing was performed on a polyacrylamide gel column containing Ampholine of pH 4.0 to 6.0 as described by Catsimpoolas.¹¹⁾ After the electrophoresis, gels were stained for protein or enzyme activity as described previously.^{1,2)}

Determination of mutarotation of the products. Mutarotation of the products from starch was determined as described previously,^{1,2)} except the pH of the reaction mixture being 8.0.

Separation and purification of F-1 and F-2. The F-1 and F-2 forms of G₄-amylase from *Pseudomonas stutzeri* NRRL-3389 were separated and purified by the method of Sakano *et al.*^{1,2)} with the following modifications: 1) after ammonium sulfate precipitation of the enzyme, the enzyme was applied on a Sephadex G-100 column (2.2 $\times$ 16 cm), equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 5 mM CaCl₂, 2) instead of DEAE-cellulose chromatography, displacement chromatography (changing pH from 8.6 to 7.0) was performed successively two times to separate and purify the two forms (F-1 and F-2), and 3) a second Sephadex G-100 chromatography (pH 8.0) was adopted for taking Polybuffer™ 96 off the active fractions eluted from the second Polybuffer exchanger™ 94 column.

RESULTS

Separation and purification of F-1 and F-2 from P. stutzeri G₄-amylase

According to the previously described method^{1,2)} G₄-amylase of *P. stutzeri* NRRL-3389 was partially purified by adsorption on starch granules and chromatography on Sephadex G-100. The enzyme obtained was subjected to chromatofocusing on a Polybuffer exchanger PBE™ 94 column (Fig. 1). Two active fractions, from No. 60 to 66 (F-1) and from No. 88 to No. 99 (F-2), were separately collected and concentrated. The fractions were separately further purified by a second Polybuffer exchanger PBE™ 94 column chromatography (Fig. 2). Each active fraction, F-1 (fraction Nos. 59–78, Fig. 2-A) and F-2 (fraction Nos. 88–103, Fig. 2-B), was concentrated and separately chromatographed on a second Sephadex G-100 column to remove the Polybuffer™ 96 (Fig. 3). The active fractions from No. 10 to No. 31 (Fig. 3-A) and from No. 9 to No. 28 (Fig. 3-B) were concentrated and used in the following experiments as the purified enzymes, F-1 and F-2, respectively. A summary of the enzyme purification is shown in Table I.

Electrophoreses of the purified enzymes, F-1 and F-2, are shown in Fig. 4. Both F-1 and

F-2 showed a single band on PAGE and SDS-PAGE.

Effects of pH and temperature on the activities of F-1 and F-2

A pH-activity curve of F-1 was coincident with that of F-2 as shown in Fig. 5. The optimum pH of the two forms was in the range

of 7.8~8.2. F-1 and F-2 were stable at pHs from 5.5 to 11.0 on standing at 30°C for 60 min in the presence of 5 mM CaCl_2 , but both were unstable at pHs lower than 6.5 and higher than 8.5 in the absence of Ca^{2+} (Fig. 5). The optimum temperature of those forms estimated for the reaction time of 5 min at pH 8.0 was 45°C. Each active form was stable below 40°C on the treatment at pH 8.0 for 60 min in the presence or absence of 5 mM CaCl_2 , but completely lost its activity on heating at 55°C ($-\text{Ca}^{2+}$) or 60°C ($+\text{Ca}^{2+}$) (Fig. 6).

FIG. 1. First Column Chromatofocusing.

The concentrate of active fractions eluted from the first Sephadex G-100 column was applied on a Polybuffer exchanger PBETM 94 column (1.0×31 cm) equilibrated with 25 mM Tris-HCl buffer (pH 8.6) containing 5 mM CaCl_2 . Elution was performed with 7.7% PolybufferTM 96-HCl buffer (pH 7.0) containing 5 mM CaCl_2 at a flow rate of 15 ml/hr and 2 ml fractions were collected. The horizontal bar fractions were separately collected and concentrated. ○, amylase activity; —, absorbance at 280 nm; ----, pH.

Molecular weight and isoelectric point of F-1 and F-2

The molecular weight of F-1 and F-2 estimated by the Weber and Osborn method¹⁰⁾ was similarly 5.5×10^4 (not shown here). The isoelectric points of F-1 and F-2 were estimated to be 5.6 and 5.3, respectively, by the method of Catsimpoolas.¹¹⁾ On the other hand, the isoelectric points of F-1 and F-2 estimated by column chromatofocusing were 7.8 and 7.4 (Figs. 1 and 2).

Effects of metal ions and chemical reagents on the enzymatic activities.

These purified forms were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA overnight and then dialyzed

FIG. 2. Second Column Chromatofocusing.

The F-1 and F-2 fractions in Fig. 1 were separately applied on the individual columns of a Polybuffer exchanger PBETM 94 (1.0×31 cm) equilibrated with the same Tris-HCl buffer as in Fig. 1. Elution was performed similarly as described in Fig. 1, and 2 ml fractions were collected. The horizontal bar fractions were collected and concentrated. A, purification of F-1; B, purification of F-2. ○, amylase activity; —, absorbance at 280 nm; ----, pH.

FIG. 3. Second Chromatography on Sephadex G-100.

F-1 and F-2 in Fig. 2 concentrated were separately applied on a Sephadex G-100 column (1.2×11 cm) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 5 mM CaCl_2 . Elution was performed with the same buffer. The horizontal bar fractions were collected and concentrated. $\circ$, amylase activity; —, absorbance at 280 nm; ----, absorbance at 254 nm.

TABLE I. PURIFICATION OF *Pseudomonas stutzeri* G_4 -AMYLASE

Purification step	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg)	Purification factor
Culture supernatant	21.5	6.5	0.3	1
Starch adsorption	0.50	12.5	25.1	84
Ammonium sulfate precipitation	0.85	56.2	61.7	206
Sephadex G-100 column (I)	0.16	12.7	80.4	268
PBE TM 94 column (I)				
F-1 fraction	0.72	71.6	88.9	296
F-2 fraction	0.47	42.5	88.5	295
PBE TM 94 column (II)				
F-1 fraction	3.38	319	94.5	315
F-2 fraction	1.66	171	103	342
Sephadex G-100 column (II)				
F-1 fraction	0.77	80.0	104	346
F-2 fraction	0.73	74.2	102	340

against 20 mM Tris-HCl (pH 8.0) overnight before use in the experiments with metal ions. After the dialyzed enzyme ($3 \sim 4 \times 10^{-2} \mu\text{M}$) had been incubated with 1 mM metallic chloride (30°C, 30 min), the residual activities of the enzymes were determined as described in MATERIALS AND METHODS. The results are shown in Table II. The activities of F-1 and F-2 were similarly inhibited by 1 mM Cu^{2+} , Fe^{2+} , Hg^{2+} and Zn^{2+} , the residual activities of F-1 and F-2 being 8, 4 $\sim$ 2, 0 and 6 $\sim$ 4% of the control, respectively.

After treatment with 1 mM EDTA at 30°C for 30 min, purified F-1 or F-2 ($4 \times 10^{-1} \mu\text{M}$)

was incubated with 0.1 mM chemical reagents (pH 6.5 or 8.0, 30°C, 90 min), and then the residual activity of F-1 or F-2 was determined as described above. NBS (both of pH 6.5 and 8.0) inhibited F-1 and F-2 activities to the degree 0 $\sim$ 10% of the control, but the other chemical reagents tested scarcely inhibited the two activities except PCMB (pH 6.5) (Table III).

Inhibition by microbial inhibitors

F-1 and F-2 ($3 \sim 4 \times 10^{-2} \mu\text{M}$) were incubated with microbial α -amylase inhibitors ($10 \sim 20 \mu\text{g/ml}$) or CDs (20 mg/ml) at 30°C for

FIG. 4. Polyacrylamide Gel Electrophoreses of F-1 and F-2 Eluted from the Second Sephadex G-100 Column.

Electrophoresis was performed with 7.5% gel with (column 7) or without (columns 1~6) 0.1% SDS; enzymes analyzed, mixture of F-1 and F-2 (columns 1, 2 and 7), F-1 (columns 3 and 4) and F-2 (columns 5 and 6); staining after the electrophoresis, 0.4% Coomassie Brilliant Blue (columns 1, 3, 5 and 7) or by iodine-starch reaction (columns 2, 4 and 6).

FIG. 5. Effects of pH on F-1 and F-2 of *Pseudomonas stutzeri* G₄-Amylase.

○, F-1 form; ●, F-2 form. —, relative activity at various pH (30°C, 5 min) based on the activity at pH 8.0; —, —, —, residual activity after incubation (30°C, 60 min) at various pHs in the presence and absence of Ca²⁺, respectively.

30 min and then the residual activities of both forms were determined as described above. The results are shown in Table IV. Typical Lineweaver-Burk plots in the presence of S-AI F-1 or F-2¹²⁾ are shown in Fig. 7. S-AI F-1 and F-2 as well as Adiposin (TAI)^{13,14)} and crude AI¹⁵⁾ non-competitively inhibited both forms

FIG. 6. Effects of Temperature on F-1 and F-2 of *Pseudomonas stutzeri* G₄-Amylase.

○, F-1 form; ●, F-2 form. —, relative activity at various temperatures (pH 8.0, 5 min) to 100% of activity at 45°C; —, —, —, residual activity after the treatment (pH 8.0, 60 min) at various temperatures in the presence and absence of Ca²⁺.

TABLE II. EFFECTS OF METAL IONS ON ENZYME ACTIVITIES OF F-1 AND F-2

Concentration of metal ions and EDTA, 1 mM. All the metal ions were added as chloride salts.

Added substance	Residual activity (%)	
	F-1	F-2
None	100	100
Al ³⁺	71	70
Ba ²⁺	93	97
Ca ²⁺	100	91
Co ²⁺	53	53
Cu ²⁺	8	8
Fe ²⁺	4	2
Fe ³⁺	25	24
Hg ²⁺	0	0
Ni ²⁺	38	33
Mg ²⁺	92	89
Mn ²⁺	86	92
Sr ²⁺	88	92
Zn ²⁺	6	4
EDTA	104	102

of G₄-amylase. CDs, nojirimycin¹⁶⁾ and S-GI (deoxynojirimycin)¹⁷⁾ had no inhibitory effect on either activity. *K_i* values for microbial α -amylase inhibitors are shown in Table V.

Hydrolyses of starch, glycogen and malto-oligosaccharides

1) Anomeric form of the hydrolysis prod-

TABLE III. EFFECTS OF CHEMICAL REAGENTS ON ENZYME ACTIVITIES OF F-1 AND F-2

Concentration of chemical reagents, 0.1 mM. The molar ratio of enzyme to chemical reagent, 1:250.

Added substance	Residual activity (%)	
	F-1	F-2
None	100	100
DTT (pH 6.5)	94	108
(pH 8.0)	101	102
DTNB (pH 6.5)	96	100
(pH 8.0)	104	102
IAA (pH 6.5)	100	95
(pH 8.0)	86	92
PCMB (pH 6.5)	78	82
(pH 8.0)	102	98
EFA (pH 6.5)	93	109
(pH 8.0)	106	101
NBS (pH 6.5)	0	5
(pH 8.0)	7	12
PMSF (pH 6.5)	99	95
(pH 8.0)	95	85
2,3-Butanedione (pH 6.5)	103	104
(pH 8.0)	97	102

TABLE IV. EFFECTS OF AMYLASE INHIBITORS ON ENZYME ACTIVITIES OF F-1 AND F-2

Inhibitor	Concentration ($\mu\text{g/ml}$)	Relative activity (%)	
		F-1	F-2
None		100	100
Adiposin (TAI)	10	15	16
Crude AI	10	29	30
S-AI F-1	10	5	5
S-AI F-2	10	5	6
S-GI	20	89	92
Nojirimycin	20	92	102
α -CD	2×10^4	95	99
β -CD	2×10^4	95	97
γ -CD	2×10^4	92	94

ucts from starch. The mutarotation of products was examined as described in MATERIALS AND METHODS. In the cases of both F-1 and F-2, a decrease in the optical rotation was similarly observed at each interval (not shown here) as reported previously,^{1,2} clearly showing that F-1 and F-2 produced α -anomer products from starch.

2) Action patterns on various substrates.

FIG. 7. Lineweaver-Burk Plots of the Enzyme Activities in the Presence and Absence of Inhibitors.

Concentration of substrate is expressed as glucose equivalents. A, hydrolysis of short chain amylose (DP=17) by F-1 form; B, hydrolysis of short chain amylose by F-2 form. ●, substrate alone; ○, with 0.07 $\mu\text{g/ml}$ of S-AI F-1; △, with 0.07 $\mu\text{g/ml}$ of S-AI F-2.

TABLE V. INHIBITION TYPE AND KINETIC CONSTANTS OF MICROBIAL AMYLASE INHIBITORS ON THE ENZYME ACTIVITIES OF F-1 AND F-2

Inhibitor	F-1		F-2	
	Inhibition type	K_i ($\mu\text{g/ml}$)	Inhibition type	K_i ($\mu\text{g/ml}$)
Adiposin (TAI)	N	1.8	N	1.6
Crude AI	N	3.3	N	3.1
S-AI F-1	N	0.15	N	0.17
S-AI F-2	N	0.18	N	0.22

The action of F-1 and F-2 on starch, shellfish glycogen, or oyster glycogen was examined under the same conditions as described previously,^{1,2} except pH of the reaction mixture being 8.0. The products identified by paper chromatography were coincident with those of the preceding papers.^{1,2} Also two dimensional paper chromatograms showing the independent action of F-1 and F-2 on non-labeled and reducing-end-labeled maltooligosaccharides (not shown here) exactly corresponded with the two dimensional paper chromatograms presented previously.^{1,2}

The enzyme (11.6 units, 7 μM) was incubated with α -, β - or γ -CD (20 mg, 0.67%) at 30°C for 1 hr. After hydrolysis of the products by glucoamylase, the glucose produced was determined by the glucose oxidase method.¹⁸ It was recognized that F-1 and F-2 could not hy-

TABLE VI. K_m AND k_o VALUES OF F-1 AND F-2

Substrate	K_m (mM)		k_o^a (1/sec)	
	F-1	F-2	F-1	F-2
Soluble starch	5.9 ^b	5.7 ^b	130	130
Oyster glycogen	7.1 ^b	6.7 ^b	190	190
Shellfish glycogen	5.9 ^b	5.9 ^b	150	150
Amylose (DP=17)	0.32	0.32	130	130

^a Obtained as $V_{\max}/e_o$, where e_o was the molar concentration of enzyme.

^b Glucose equivalents.

drolyze α - and β -CDs and could scarcely cleave γ -CD (the degree of hydrolysis, less than 0.07%).

3) *Michaelis constants and molecular activities on various substrates.* K_m and k_o values of these forms (F-1 and F-2) for short chain amylose (DP=17), soluble starch, shellfish glycogen and oyster glycogen were calculated from the Lineweaver-Burk plots and are summarized in Table VI.

DISCUSSION

In the previous papers^{1,2)} only the F-2 form of *Pseudomonas stutzeri* G₄-amylase was separated and purified with a column of DEAE-cellulose. In the present paper partially purified enzyme eluted from first Sephadex G-100 column was further purified through a novel exchanger column, a Polybuffer exchanger PBETM 94 column. As a result the F-1 and F-2 forms were separated completely and purified electrophoretically to a homogeneous state, so this column chromatofocusing method^{4,5)} is effective in separating as well as purifying each active form of *P. stutzeri* G₄-amylase.

F-1 and F-2 showed the same optimum pH at 8.0, and the same pH activity curve, having no shoulder near pH 6.0 (Fig. 5). On the other hand, the optimum pH of F-2 estimated previously^{1,2)} was 6.0~6.5, and the optimum pH of the partially purified enzyme²⁾ as well as Robyt and Ackerman's enzyme³⁾ was 8.0~8.5, having a shoulder near pH 6.0. These discrepancy in the optimum pH may be due to the

different conditions of enzyme assay. In this study enzyme reactions, especially at weakly acidic pH, were exactly and carefully pursued; the enzyme reactions were stopped by directly adding 1 N acetic acid to the reaction mixtures and by heating the mixture in a boiling water bath for 5 min, instead of directly adding the Somogyi reagent to the mixture, then the rate of the reactions being determined by using the Nelson-Somogyi method.⁷⁾

The isoelectric points of F-1 and F-2 were estimated by the method of Catsimpoalas¹¹⁾ to be 5.6 and 5.3, respectively, and the molecular weights of F-1 and F-2 were 5.5×10^4 (SDS-PAGE test), the values being a little smaller than the value of 5.7×10^4 obtained before.^{1,2)} Both F-1 and F-2 acted on starch from the non-reducing end to produce maltotetraose of the α -anomeric form. This fact confirmed that this amylase should be classified as an *exo*- α -amylase. Action patterns of F-1 and F-2 on starch, glycogen and maltooligosaccharides were paper chromatographically and kinetically very similar to those of previous research.^{1,2)}

F-1 and F-2 scarcely cleaved any α -1, 4-glucosidic linkages of α -, β - and γ -CDs under the extreme experimental conditions above. On the other hand so-called α -amylases (endo-amylases), *i.e.*, Taka-amylase A and bacterial saccharifying amylase, can hydrolyze CDs.¹⁹⁻²¹⁾ From these results, it would safely be stated that the inability to attack CDs is the common feature of *exo*-amylases, since other *exo*-amylases, *i.e.*, glucoamylase, β -amylase and maltohexaose-forming amylase from *Klebsiella pneumoniae*, are also reported not to attack CDs.²²⁻²⁴⁾

Because of the different conditions of enzyme assay from those described before,^{1,2)} various effects of metal ions, chemical reagents and amylase inhibitors on enzymatic activities were a little different from those of preceding studies.^{1,2)} But these effects on F-1 and F-2 as described above were similar, as follows: 1) in addition to Cu^{2+} and Hg^{2+} , Fe^{2+} and Zn^{2+} (1 mM, 30°C, 30 min) inhibited markedly the activities of F-1 and F-2 ($3 \sim 4 \times 10^{-2} \mu\text{M}$), 2)

only NBS (0.1 mM, pH 6.5 and 8.0) had an inhibitory effect on F-1 and F-2 ($4 \times 10^{-1} \mu\text{M}$), while in the previous reports 2,3-butanedione as well as NBS (1 mM) inhibited F-2 ($3 \times 10^{-2} \mu\text{M}$), 3) Ca^{2+} stabilized F-1 and F-2 at alkaline pH, 4) α -, β - and γ -CDs did not inhibit F-1 and F-2 activities and 5) Adiposin (TAI), crude AI, and S-AI F-1 and F-2 non-competitively inhibited F-1 and F-2 activities, their K_i for F-1 and F-2 being similar, 1.8~1.6, 3.3, 0.15~0.17 and 0.18~0.22 $\mu\text{g/ml}$, respectively.

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