Purification and Characterization of β-Amylase from Ginseng

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β-Amylase activity was found in ginseng for the first time. β-Amylase was purified to apparent homogeneity on polyacrylamide slab gel from ginseng powder by dialysis of the crude extract and by column chromatography on diethylaminoethyl (DEAE)-Sephadex A-50, Sephadex G-100 and hydroxyapatite. The β-amylase was most active at pH 5.0 and 50 °C. The molecular weight of the enzyme was estimated to be 63 kilodaltons (kDa) by both Sephadex G-100 column chromatography and sodium dodecyl sulfate gel electrophoresis. The isoelectric point of the enzyme was pH 5.3. Amino acid analysis indicated that the enzyme was composed of 512 amino acid residues. The content of neutral sugars was 8.5%, and no amino sugar was detected.

Keywords β-amylase; ginseng; amino acid composition; isoelectric point; gel filtration

Panax ginseng C. A. MEYER has been administered to weak or ill-fed patients as a nutrient drug in many prescriptions of herbal medicine and treated not only as a tonic but even as the "elixir of life." In spite of its prolonged appraisal, relatively little scientific research has been done on ginseng as a drug. Recently, ginsenosides, which are believed to be the effective components of ginseng saponin, were isolated and their structures were determined.[1] But several pharmacological effects not due to saponin are found in the water or alcohol extract.[2] Therefore, it is likely that other bioactive ingredients exist in ginseng which have not been identified yet.

Previously, the authors reported that some digestive enzyme activities were inhibited by adding various crude drugs.[3] On the other hand, *Glycyrrhiza uralensis* and *Panax ginseng* activated the amylase activity of diastase at relatively low pH. The stimulation was more profound with ginseng than glycyrrhiza.

β-Amylase is widely distributed in plants, and has been isolated and purified from several plant tissues such as soybean,[4] barley,[5] radish,[6] taro[7] and sweet potato.[8] However, no paper has been reported on β-amylase in a crude drug such as ginseng which has been processed and preserved for a long time. In this study, we isolated and purified β-amylase from *Panax ginseng* for the first time, and investigated its enzymatic properties and amino acid composition.

Experimental

Purification of Crude Enzyme Twenty grams of Japanese Pharmacopeial (JP) ginseng powder (*Panax ginseng*) was extracted with 200 ml of 20 mM acetate buffer (pH 5.0) at room temperature for 1 h. The crude extract was centrifuged at 3000 rpm, at 4 °C for 30 min. The supernatant was dialyzed against 20 mM acetate buffer (pH 5.0) at 4 °C for 4 d.

Enzyme Assay a) β-Amylase Activity: One milliliter of enzyme solution was added to 1 ml of 1% soluble starch containing 0.1 M acetate buffer (pH 5.0), and the mixture was incubated at 40 °C for 10 min. The amount of reducing sugars produced was determined colorimetrically by the Somogyi–Nelson method.[9] One unit of enzyme activity was defined as the amount which catalyzed the formation of 1 μmol of maltose per min.

b) α-Amylase Activity: One milliliter of enzyme solution was added to 1 ml of 1% soluble starch containing 0.1 M acetate buffer (pH 5.0), and the mixture was incubated at 40 °C for 10 min. The enzyme reaction was stopped with 4 ml of 10 mM iodine solution containing 60 mM KI and 0.01 M HCl. The blue value was determined spectrophotometrically at 660 nm.

c) The other enzyme assays were described in previous papers[3e,10]

Disk-Gel Electrophoresis Homogeneity of the enzyme preparation was examined by disk electrophoresis using 7.5% polyacrylamide gel in Tris–HCl buffer (pH 9.4), according to the method of Davis.[11]

Paper Chromatography The identification of the hydrolysis product of starch was performed by paper chromatography using Toyo No. 50 filter paper (40 × 40 cm) with 6:4:3 (v/v) 1-butanol–pyridine–water for development, and sugar spots were detected by means of the dip method.[12]

Optical Rotation of Sugars Produced from Starch To determine the anomeric type of the products, the changes in the optical rotation during the enzyme reaction were monitored using a Horiba SEPA-200, according to the method of Fuwa and Nikuni.[13]

Determination of Isoelectric Point Isoelectric focusing was performed by the method of Matsuo and Horio,[14] by the use of an LKB electrofocusing column 8100 (110 ml capacity) with carrier Ampholites (pH 3.5—10, LKB-Producter, Sweden) for 40 h at 400 V, at 4 °C.

Determination of Molecular Weight a) Gel Filtration: In order to determine the molecular weight of the enzyme, gel filtration was performed using a Sephadex G-100 column (2 × 120 cm). The gel was equilibrated with 10 mM phosphate buffer (pH 6.0) containing 0.1 M sodium chloride and 1 mM mercaptoethanol. One milliter of enzyme solution containing the following standard proteins was charged on the column and eluted with the same buffer: catalase (MW: 232 kilodaltons (kDa)), aldolase (158 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13 kDa), from Sigma Chemical Company (U.S.A).

b) Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis: To determine the subunit molecular weight, SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[15] Prepared enzyme solution and the following solution containing standard proteins were applied to the gel: rabbit muscle phosphorylase b (MW: 97 kDa), BSA (66.2 kDa), hen egg white albumin (42.7 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa), from Bio-Rad Laboratories (U.S.A.). The protein band on the gel was dyed by use of 0.25% Coomassie brilliant blue R-25 (CBB) solution containing 50% methanol and 10% acetic acid. Schiff's reagent was used in order to dye the sugars on the gel.

Amino Acid Analysis Lyophilized enzyme preparation was hydrolyzed with 6 N hydrochloric acid in evacuated, sealed tubes at 110 °C for 24, 48 and 72 h. Amino acid analysis was performed by means of a Shimadzu LC-3A automatic amino acid analyzer. Independent analysis of cystine and cysteine was performed with performic acid oxidation according to the method of Hirts.[16] Tyrosine and tryptophan were determined by the spectrophotometric method of Goodwin and Morton.[17]

Analysis of Sugars in the Enzyme a) Neutral Sugars: Lyophilized enzyme preparation was hydrolyzed with 2 N trifluoroacetic acid (TFA) in evacuated, sealed tubes at 100 °C for 6 h. The hydrolyzates were then converted to alditol acetates as described by Sawardeker *et al.*[18] and analyzed by gas-liquid chromatography (1% Silar 10 C column).

b) Amino Sugars: Lyophilized enzyme preparation was hydrolyzed in the same manner as neutral sugars. The hydrolyzed sugars were then adsorbed on a column of Dowex 50 W × 8 (H^+ form) followed by elution

TABLE I. Effects of Glycyrrhiza and Ginseng on Various Digestive Enzymes

	Residual enzyme activity (%)[a]							
Activity tested	Starch saccharifying		Starch dextrinizing		Protein peptic		Lipid peptic	Cellulose saccharifying
Enzyme source (Conc. of sol.)	Diastase (0.1%)	Pancreatin (0.05%)	Diastase (0.05%)	Pancreatin (0.05%)	Biodiastase (0.05%)	Pancreatin (0.05%)	Pancreatin (1.0%)	Biodiastase (0.1%)
Sample[b] pH	5.0	7.0	5.0	7.0	3.0	8.5	8.0	4.5
Control[e]	100	100[c]	100	100[c]	100	100	100[d]	100[c]
Glycyrrhiza	122	62[c]	118	105[c]	96	69	79[d]	88[c]
Ginseng	149	56[c]	122	96[c]	101	96	100[d]	108[c]

a) Enzyme activities were determined by the methods described in Experimental. *b*) Five mg/ml of crude drug was added to the enzyme solution except for *c*) (10 mg/ml) and *d*) (1 mg/ml). *e*) No crude drug was added.

TABLE II. Comparison of Plant β-Amylase Activities

Plant	β-Amylase (units/g dry wt.)
Ginseng	170
Soybean	430
Japanese radish	2.7
Taro	350
Sweet potato	290

Amylase activity was determined by the method described in Experimental.

with 2 N hydrochloric acid. N-Acetylation of the hydrolyzates was performed with sodium bicarbonate and acetic anhydride. They were then converted to alditol acetates and analyzed by gas-liquid chromatography.

Results

β-Amylase from Ginseng Table I summarizes the effects of JP glycyrrhiza (*Glycyrrhiza uralensis*) and JP ginseng (*Panax ginseng*) on the activities of various digestive enzymes. It is particularly noteworthy that starch saccharifying activity increased to about 150% of the control value (without crude drug) on adding ginseng.

In order to find the cause of this activation, JP ginseng on the market was extracted and dialyzed. The dialysate showed a high amylase activity. Soybean, radish, taro and sweet potato, which are known to contain large amounts of β-amylase were extracted and dialyzed in the same manner as ginseng, and the β-amylase activities obtained were compared with each other. As shown in Table II, β-amylase activity was relatively high in ginseng tissue.

Purification of Ginseng β-Amylase Four hundred milliliters of dialyzed solution that had originated from 40 g of ginseng were charged on a diethylaminoethyl (DEAE)-Sephadex A-50 column (2.8 × 24 cm) equilibrated with 20 mM acetate buffer (pH 5.0). The column was washed with the same buffer and the adsorbed enzyme was eluted from the column with a linear gradient of sodium chloride from 0 to 1 M in the same buffer. The active fractions were collected and rechromatographed on DEAE-Sephadex A-50 in the same way. The active fractions were pooled and dialyzed against 10 mM phosphate buffer at 4 °C for 2 d. The dialyzed solution was concentrated to 5 ml with polyethyleneglycol. The enzyme solution was applied to a Sephadex G-100 column (2 × 120 cm) previously equilibrated with 10 mM phosphate buffer (pH 6.0) containing 0.1 M sodium chloride and 1 mM mercaptoethanol. Elution was carried out with the same buffer. The elution pattern is shown in Fig. 1.

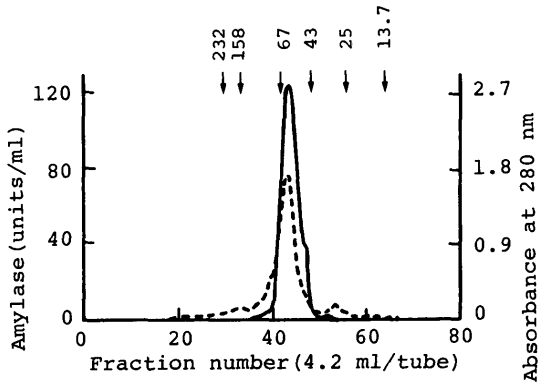

Fig. 1. Elution Profile of Ginseng β-Amylase on Sephadex G-100 Column Chromatography

——, amylase activity; ------, absorbance at 280 nm. Catalase (MW: 232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) were used as molecular weight standards.

TABLE III. Purification Procedure for Ginseng β-Amylase

Procedure	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	400	5230	10.8	100
DEAE-Sephadex A-50	65	2326	22.8	44.5
DEAE-Sephadex A-50 (rechromatography)	50	1758	34.6	33.6
Sephadex G-100	25	1707	81.5	32.6
Hydroxyapatite	51	527	130	10.1

Fractions from 41 to 46 were combined and dialyzed against water at 4 °C overnight. The dialyzed solution was charged on a hydroxyapatite column (1.5 × 10 cm) previously equilibrated with 5 mM phosphate buffer (pH 6.5) containing 1 mM mercaptoethanol. The column was thoroughly washed with the same buffer and the adsorbed enzyme was eluted with a linear gradient of phosphate buffer (pH 6.5) from 5 mM to 0.2 M containing 1 mM mercaptoethanol. The active fractions were collected and dialyzed against water at 4 °C overnight. The purification steps are summarized in Table III. Homogeneity of the purified enzyme preparation was tested by disk-gel electrophoresis with polyacrylamide gel. As shown in Fig. 2, the enzyme preparation was considered to be homogeneous.

Hydrolysis of Starch by Starch by Ginseng β-Amylase a) The Relationship between the Iodine Color Reaction (Blue

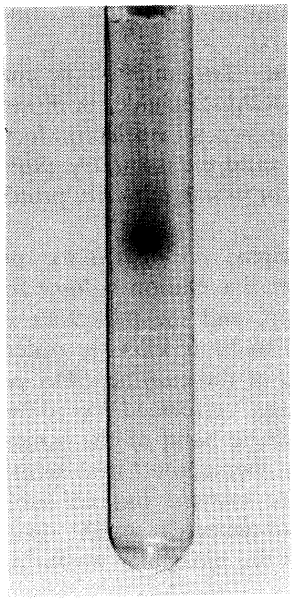

Fig. 2. Polyacrylamide Gel Electrophoresis Pattern of Ginseng β-Amylase

The purified enzyme solution (30 μl) containing 10 μg of protein was subjected to electrophoresis on a column at pH 9.4.

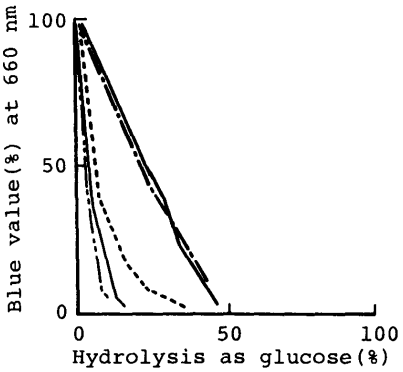

Fig. 3. Hydrolysis of Starch by Amylases from Ginseng and Other Plants

——, ginseng β-amylase; — · —, sweet potato β-amylase; ------, taka-diastase from *Aspergillus oryzae*; ——, diastase from barley; — -- —, α-amylase from *Bacillus subtillis*.

Value) and the Degree of Hydrolysis of Starch: The purified enzyme was incubated with 0.2% soluble starch solution and the degree of hydrolysis and the blue value at 660 nm were investigated in comparison to those with several other amylases. As shown in Fig. 3, a linear relationship was recognized between the degree of hydrolysis and the blue value for β-amylase from ginseng, as in the case of sweet potato β-amylase.

b) Products from Starch: One unit if the enzyme solution was incubated with 2% substrate solution at 40 °C. After 5, 10, 20, 40, 60, 120 and 180 min and 24 h, 10 μl of the reaction mixture was spotted on paper and chromatography was performed. The only oligosaccharide found was maltose at each time interval. The reaction mixture after 24 h gave a red-purple color with iodine and the yield of maltose was 47%.

Anomeric Type of the Reaction Product The enzyme solution was incubated with 5% soluble starch solution at

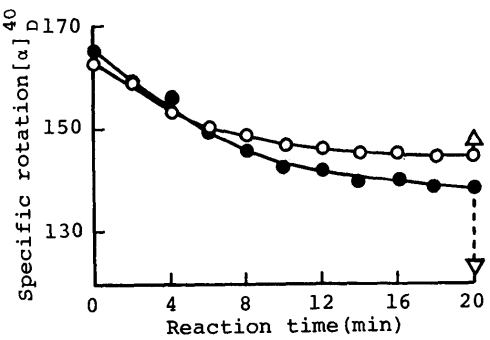

Fig. 4. Changes in Specific Rotation During Enzymatic Hydrolysis of Soluble Starch

The experimental conditions are described in the text. ○, ginseng β-amylase; ●, taka-α-amylase; △, change and extent of optical rotation.

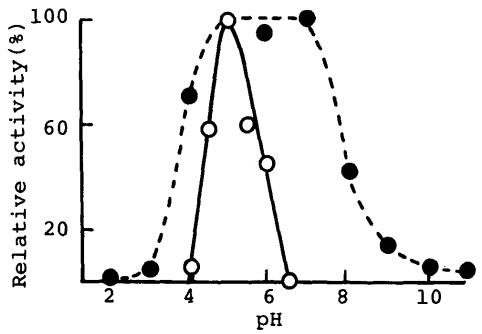

Fig. 5. Optimum pH and pH-Stability of Ginseng β-Amylase

Optimum pH (○): The reaction mixtures, consisting of 500 μl of the enzyme solution, 1 ml of 1% soluble starch and 500 μl of 0.2 M acetate buffer of various pHs, were incubated at 40 °C for 10 min. pH-Stability (●): The enzyme solution (250 μl) was mixed with 250 μl of 20 mM acetate buffer of various pHs and incubated at 40 °C for 1 h. Then 500 μl of 0.2 M acetate buffer and 1 ml of 1% soluble starch were added and the activity was measured in the usual way.

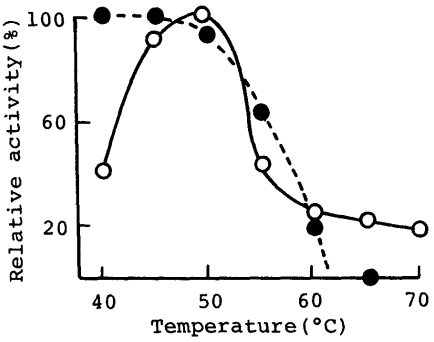

Fig. 6. Optimum Temperature and Thermostability of Ginseng β-Amylase

Optimum temperature (○): The activity was assayed in the usual manner except for the incubation temperature. Thermostability (●): The activity was measured in the usual way at 40 °C after incubation of the enzyme solution at various temperature for 10 min.

40 °C and the changes in the optical rotation with time were examined, according to the method of Fuwa and Nikuni[13] (Fig. 4). The enzyme reaction was stopped by adding sodium carbonate. Mutarotation of the products was decreased in the case of the enzyme from ginseng, but was increased with taka-α-amylase. Hence, the anomeric type of hydrolyzates formed by ginseng amylase was found to be β-anomer.

Effect of pH and Temperature on Ginseng β-Amylase Optimum pH and the pH-stability of the enzyme

are shown in Fig. 5. The optimum pH was at 5.0. To examine the pH-stability of the enzyme, the enzyme was incubated at 40 °C for 1 h in acetate buffers of various pHs, and the residual activity was measured at pH 5.0. The enzyme was stable over the range of pH 5—7.

Optimum temperature and thermostability of the enzyme are shown in Fig. 6. The optimum temperature was at 50 °C under the reaction conditions employed (reaction for 10 min). To measure the thermostability of the enzyme, the enzyme was incubated at pH 5.0 at various temperatures for 10 min and the remaining activity was determined by the standard method. Over 91% of the enzyme activity remained at temperatures below 50 °C, but the enzyme was inactivated at 65 °C.

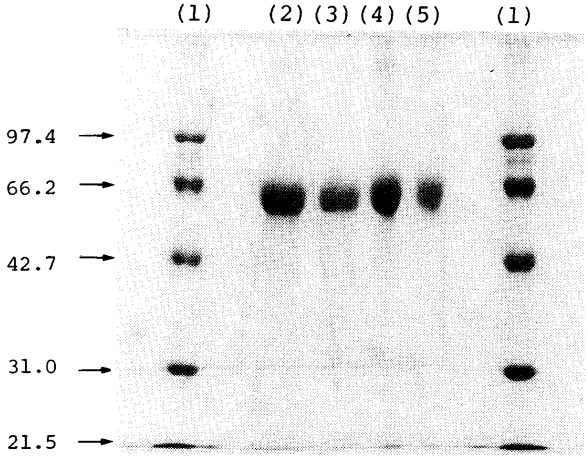

Fig. 7. SDS-Polyacrylamide Gel Electrophoresis Pattern of Ginseng β-Amylase

(1) Molecular weight standards; (2) 7.5 μg of the enzyme without SDS and mercaptoethanol treatment; (3) 15 μg of the enzyme treated in the same way as (2); (4) 7.5 μg of the enzyme with SDS and mercaptoethanol treatment; (5) 15 μg of the enzyme treated in the same way as (4). Rabbit muscle phosphorylase b (MW: 97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa) were used as molecular weight standards.

TABLE IV. Effects of Metal Ions and Chemical Reagents on Ginseng β-Amylase

Metal or chemical reagent	Concentration (mM)	Residual activity (%)
None	—	100
NaCl	10	94
$CaCl_2$	10	96
$BaCl_2$	10	83
$MgCl_2$	10	81
$MnCl_2$	10	106
$CoCl_2$	1	79
$NiCl_2$	1	68
$ZnCl_2$	1	70
$FeCl_2$	1	32
$HgCl_2$	1	13
$PbCl_2$	1	16
$CuCl_2$	1	17
$SnCl_2$	1	31
Iodoacetic acid	1	66
Dithiothreitol	1	95
PCMB	0.1	23
EDTA	1	80
N-Ethylmaleimide	1	74

The reaction mixtures, consisting of 1 ml of 1% soluble starch and 0.2 ml of reagent at 10 times the concentration shown in the table, were incubated at 40 °C for 10 min. Then, 0.8 ml of the enzyme solution was added and the activity was measured in the usual way. PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

Isoelectric Point Ten units of the enzyme preparation fractionated by DEAE-Sephadex A-50 column chromatography were charged on an isoelectric focusing column in a glycerol gradient, and the isoelectric point was determined to be 5.3. Most coexisting impurity proteins were removed by the procedure.

Molecular Weight A logarithmic plot of molecular weight against relative elution volume from Fig. 1 gave a linear relationship, and the molecular weight of the enzyme was found to be 63 kDa. In order to elucidate the subunit structure of the enzyme, SDS–polyacrylamide gel electrophoresis was performed. As shown in Fig. 7, the enzyme gave a single band of the same mobility with or without SDS or mercaptoethanol treatment. The result indicated that the enzyme has no subunit structure. A logarithmic plot of molecular weight against relative mobility indicated that the molecular weight of the enzyme was 63—64 kDa.

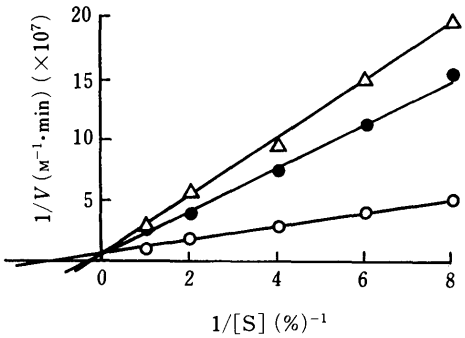

Fig. 8. Lineweaver–Burk Plots of Ginseng β-Amylase in the Presence of Cyclodextrins

○, control; ●, 0.2 mM cyclohexaamylose; △, 5 mM cycloheptaamylose.

TABLE V. Amino Acid Composition of Ginseng β-Amylase

Amino acid	Moles of amino acid	
	Per 63000 g protein[a]	Nearest integer
Aspartic acid	59.5	60
Threonine	19.3[b]	19
Serine	33.4[b]	33
Glutamic acid	61.6	62
Proline	31.4	31
Glycine	37.1	37
Alanine	32.2	32
Cysteine	1.5[c]	2
Valine	33.5	34
Methionine	12.3	12
Isoleucine	20.9	21
Leucine	57.7	58
Tyrosine	27.0[d]	27
Phenylalanine	23.9	24
Histidine	9.4	9
Lysine	27.0	27
Arginine	15.7	16
Tryptophan	7.7[d]	8
Total		512

a) Values of about 8.5% sugar per 63000 g of enzyme were determined by phenol–sulfuric method. *b*) Extrapolated to zero time from 24, 48 and 72 h hydrolyzates. *c*) For 18 h hydrolyzates after performic acid oxidation. *d*) Method of Goodwin and Morton.[17]

TABLE VI. Comparison of Properties of Plant β-Amylase

Properties	Origin			
	Ginseng	Soybean	Japanese radish	Sweet potato
Optimum pH (Substrate: starch)	5.0	5—6 (37 °C)	6.0—6.5	4—5 (30 °C)
pH-stability	5—7	5—7	5.7—6.5	4—9
Molecular weight (kDa)	63	57—61.7	58—62	206 (tetramer) 50—57.2 (monomer)
$E^{1\%}_{280\,nm}$	16.7	17.3		17.1—17.7
PI	5.3	5.2—5.9	4.9	4.7—4.8
Inhibitor	PCMB, Fe, Hg, Pb, Cu, Sn		PCMB	Glucose, maltose, PCMB, Ag
K_m	0.61% starch	1.8 mM/maltotriose 0.42 mM/maltotetraose 2.5 mM/ml/amylopectin	1.1 mg/ml amylopectin	0.073 mM/amylose 0.021%/starch
K_i Cyclohexaamylose;	0.08 mM	0.35 mM		0.17 mM
Cycloheptaamylose;	1.6 mM	0.98 mM		
			PCMB; 2.58×10^{-7} M	

Influence of Metal Ions and Chemical Reagents The effects of metal ions and inhibitors on the enzyme activity were examined. Enzyme was added to the substrate solution preincubated with a reagent at 40 °C for 5 min, and residual activity (%) was determined. As shown in Table IV, enzyme activity was reduced by $FeCl_2$, $HgCl_2$, $PbCl_2$. $CuCl_2$, and $SnCl_2$. The enzyme was also inhibited by SH-inhibitors such as *p*-chloromercuribenzoic acid (PCMB), and the inhibition was abolished by adding reducing agents such as cysteine or mercaptoethanol.

Michaelis Constant (K_m) and Inhibitor Constant (K_i) The affinity of the enzyme for substrate and inhibitors was investigated. Soluble starch was used as the substrate, and cyclohexaamylose (α-CD) and cycloheptaamylose (β-CD) were used as inhibitors. As shown in Fig. 8, the kinetics of inhibition of amylase activities were estimated from Lineweaver–Burk plots using various concentrations of starch. The K_m value for the hydrolysis of starch was 0.61% and the V_{max} value was 1.1×10^{-7} (M/min). α-CD and β-CD inhibited the activity of ginseng β-amylase competitively, and the K_i values for α-CD and β-CD were 0.08 and 1.6 mM, respectively.

Amino Acid Composition and Sugar Analysis The amino acid composition of ginseng β-amylase was determined. Average values of the analytical data after 24, 48 and 72 h of hydrolysis of the enzyme are shown in Table V. Sugars were estimated by the phenol sulfuric acid method, and 8.5% of sugars (calculated as glucose) were found to exist in the enzyme. The position of the band dyed with Schiff's reagent on the polyacrylamide gel was the same as that of the protein band dyed with CBB. Alditols from glucose, galactose, mannose and arabinose were detected, but amino sugars were not detected. Considering the molecular weight of the enzyme and amount of sugars, the number of amino acid residues of the enzyme was considered to be 512.

Discussion

The relationship between blue value and the degree of hydrolysis of starch and the result of paper chromatography of products indicate that the enzyme, which we purified from ginseng, hydrolyzes starch to maltose from the non-reducing end in an exo-wise manner. It was also recognized from the mutarotation of the product that only β-maltose was produced. Similar results were obtained using crude enzyme extract, which indicates that β-amylase is the only amylase in ginseng.

The enzyme was inactivated by SH-inhibitor such as PCMB, the activity being restored by thiol compounds such as cysteine. The result indicates that an SH group exists in the molecular structure, as in other plant β-amylases. When the properties of ginseng β-amylase and other plant β-amylases were compared, little difference was found except that the ginseng enzyme has a monomeric structure while the others have polymeric structure. On column chromatography only one active peak was found, and it seemed that no isozymes of this enzyme exist in ginseng. The isoelectric point of the β-amylase seemed to be slightly lower than those of other β-amylases. The sugar content of the enzyme was 8.5%, which seems rather high compared to those of other plant β-amylases. The number of cysteine residues in the enzyme (2 mol) seemed to be smaller than those in other β-amylases (5 mol for the soybean enzyme).[19]

The ginseng used in this study was white-ginseng. The other type of ginseng called red-ginseng is steamed at high-temperature and processed. Amylase activity was not found in the red-ginseng, but the powder tasted sweet. It seems likely that the starch contained in the ginseng was saccharified by β-amylase during the processing.

As regards raw ginseng, it seems that there are differences in β-amylase contents in the center of the root, peel of the root and fine roots. Amylase is also supposed to exist in glycyrrhiza (Table I). The enzyme may also be present in other crude drugs, in particular in roots.

The effective ingredient of ginseng is usually regarded as saponin, but it is known that fat-soluble components, monosaccharides, polysaccharides, amino acids, peptides, proteins and basic substances exist in ginseng, besides saponin. The extracts with hot water amount to 30—40% of the starting material. Studies on the extraction and isolation of these substances are still at a very early stage.

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