The Sennoside Constituents of Rhei Rhizoma and Sennae Folium as Inhibitors of Serum Monoamine Oxidase

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Four (in total) compounds with inhibitory effects on the activity of bovine serum monoamine oxidase were isolated from the methanol–water extracts of Rhei Rhizoma (Daiou) and Sennae Folium (Senna), and identified as sennosides A, B, E and F. Among them, sennosides A and B were obtained from both Rhei Rhizoma and Sennae Folium, while sennosides E and F were detected only in the former. The 50% inhibitory concentrations of the four sennosides were 17, 9, 24 and 13 μM, respectively, and the type of inhibition by sennosides A and B with respect to allylamine as the substrate was non-competitive.

Keywords serum monoamine oxidase; enzyme inhibitor; sennoside; Rhei Rhizoma; Sennae Folium

Copper-containing amine oxidase (E.C. 1.4.3.6) is an enzyme which, like flavin-containing amine oxidase (E.C. 1.4.3.4), catalyzes the oxidative deamination of a variety of amines to yield the corresponding aldehydes. It is contained in the highest concentration, along with lysyl oxidase (E.C. 1.4.3.13), in mammalian (*e.g.* bovine,[1] pig,[2] rabbit,[3] rat and human[4] *etc.*) connective tissues, although a considerable amount of the enzyme, termed serum monoamine oxidase (serum MAO), is present in the blood plasma.[5-9] In the field of human clinical biochemistry, elevation of the serum MAO activity has been considered as a marker of hepatic fibrosis,[10] arteriosclerosis,[11] *etc.*, since increased biosynthesis of this enzyme is generally associated with acceleration of collagen metabolism within the corresponding organs. The precise role of the enzyme is not clear at present, but it is known that the activity of this enzyme including serum MAO is greatly reduced by various neuropsychiatric agents such as iproniazid (an anti-tuberculous drug with an anti-depressant activity),[12] phenylhydrazine (an anti-depressant drug),[13] penicillamine (an anti-Wilson's disease drug),[14] levodopa (an anti-Parkinson's disease drug)[15] and carbidopa (an inhibitor of β-3,4-dihydroxyphenylalanine (DOPA)-decarboxylase),[16] as well as by aminoacetonitrile,[17] semicarbazide,[18] *etc.* In order to obtain new biologically active substances which affect collagen metabolism and/or that some neurological activity, therefore, the authors have been carrying out screening tests of many (more than 300) crude drug extracts using inhibitory action on bovine serum MAO activity as a biochemical marker. The methanol (MeOH)–water extracts of Rhei Rhizoma (Daiou) and Sennae Folium (Senna) inhibited the enzyme activity, and as summarized in Chart 1, four (in total) compounds, I—IV, were isolated by procedures involving thin-layer chromatography (TLC) and paper chromatography (PC) with monitoring of the enzyme-inhibitory effect.[19] Among them, I and II were obtained from both Rhei Rhizoma and Senna Folium, while III and IV were detected only in the former.

Results and Discussion

The properties of I—IV seemed to resemble those of sennosides A, B, E and F described by Oshio *et al.* as components of Rhei Rhizoma, respectively.[20] As described in detail in Experimental, I and II were identified as sennosides A and B, respectively, based on a comparison of the ultraviolet (UV), infrared (IR), proton–nuclear magnetic resonance (^{1}H-NMR) and secondary-ion mass spec-

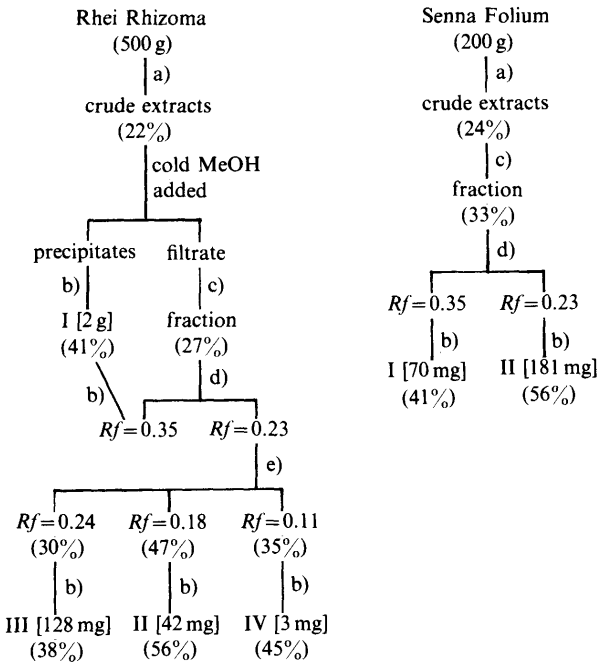

Chart 1. Isolation Procedures of the Enzyme-Inhibitory Components, I—IV, from the Crude Extracts of Rhei Rhizoma and Sennae Folium

[ ], yield; ( ), percent inhibition of examined samples (10 μg/ml).
a) The preparation procedures were described in Experimental. b) Recrystallized from 70% (v/v) aqueous acetone. c) Subjected to polyamide column chromatography with Wako C-200 using MeOH as the eluting solvent. Fractions obtained were evaporated to dryness *in vacuo* below 50°C. The contents of fractions with the enzyme-inhibitory effect were dissolved in 0.5% aqueous sodium bicarbonate, and extracted with *n*-butanol after acidification with HCl. The *n*-butanol layers were evaporated to dryness *in vacuo* below 50°C, and dissolved in MeOH. d) Subjected to TLC on silica-gel G with ethyl acetate–isopropanol–water (4 : 3 : 3, v/v) as the developing solvent. After developing, the silica gel of areas with the activity were dried, scraped off, and left in 0.5% aqueous sodium bicarbonate for 1 h at room temperature, followed by the same treatment of alkaline extracts as in the case of c). e) Subjected to PC on Whatman 3 MM with *n*-butanol–ethanol–0.2 M citrate buffer (pH 6.2) (2 : 1 : 2, v/v). The following processes were similar to those in d).

troscopy (SI-MS) spectra with those of authentic samples, and III and IV were characterized as oxalate esters of I (sennoside E) and II (sennoside F), respectively.

Various compounds related to I—IV, including sennosides C and D, in addition to a variety of monoanthraquinolic substances (*e.g.* rhein, emodin, aloe-emodin, chrysophanol, physcion and their glycosides) are contained in these two crude drugs.[20-23] However, in this investigation, only I—IV were isolated as components with the enzyme-inhibitory effect. Indeed, authentic samples of the monoanthraquinones (aglycones) mentioned above caused virtually no inhibition of the enzyme activity. The 50% inhibitory concentrations (IC_{50}) of I—IV were 17, 9 (data obtained using authentic samples of sennosides A and B, respectively), 24 and 13 μM, respectively. It is known that sennosides dissolved in solvents are unstable, suffering degradation. Therefore, it was not clear whether I—IV, dissolved in dimethylsulfoxide (DMSO) or 0.5% (v/v) aqueous sodium bicarbonate ($NaHCO_3$) and then immediately injected into the reaction mixture, were present as the intact materials during the assay period, although no differences in their IC_{50} values were observed between the use of DMSO and 0.5% (v/v) $NaHCO_3$ as the solvents. However, the facts that only the compounds with 2,2′-dicarboxyl groups in the bianthracene rings (sennosides A, B, E and F) had the enzyme-inhibitory action and that the sennosides with an erythro C_{10}–$C_{10'}$ bond (B and F) are more potent inhibitors than the stereoisomers with the threo bond (A and E) seemed to indicate that the 2,2′-dicarboxyl groups of the bianthracene compounds (especially, those with the erythro C_{10}–C_{10} bond) can chelate with functional copper of the enzyme molecule.

As shown in Fig. 1, a kinetic study varying the concentrations of both the substrate (allylamine) and the inhibitors (authentic samples of sennosides A and B) revealed no change in the K_m value (3.9×10^{-4} M), indicating that inhibition of bovine serum MAO by the sennosides with respect to allylamine as the substrate was non-competitive.

Both Rhei Rhizoma and Sennae Folium are well known as purgative crude drugs. It has also been elucidated that their purgative activity is mainly owing to the sennoside constituents[20-22] and that sennosides can express such pharmacological activity after oral administration only through the action of their degradation products in the intestine.[24] Indeed, it was reported that, in animal experiments, sennosides injected intravenously had no purgative effect.[25] However, In our investigation searching for naturally occurring substances with biochemical and/or pharmacological activities other than purgation, intact sennosides A, B, E and F exhibited the enzyme-inhibitory activity employed as a marker. Their IC_{50} values (9—24 μM) were similar to those obtained in this assay system for some known potent inhibitors (2—30 μM),[26] although animal experiments are needed in order to elucidate whether the sennosides injected as the intact forms can display such *in vivo* effects, as in the case of penicillamine (depression of collagen metabolism in connective tissues)[27] and phenylhydrazine (an anti-depressant activity),[13] *etc.*

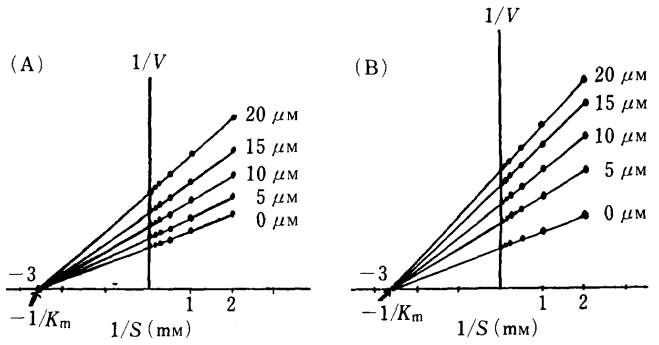

Fig. 1. Lineweaver–Burk Plot of the Activity of Bovine Serum Monoamine Oxidase in the Presence or Absence of Sennoside A (A) and B (B) with Various Concentrations of the Substrate (Allylamine) and the Inhibitors

$K_m = -1/-2.55\,\text{mM} = 3.9 \times 10^{-4}$ M.

Experimental

Apparatus All melting points are uncorrected. The UV spectra were recorded with a Hitachi 124 spectrometer. The IR spectra were run on a Jasco A-3 IR spectrometer. The ^{1}H-NMR spectra were taken by the use of a JEOL GX-500 NMR spectrometer at 500 MHz. The SI-MS spectra were obtained with a Hitachi M-80 mass spectrometer. Isotachophoresis was performed using a Shimadzu IP-2A isotachophoretic analyzer equipped with a PGD-2 potential detector and the separation was done in a PTFE tube (0.5 mm × 4 cm + 1 mm × 15 cm) maintained at 20 °C.

Materials and Chemicals Powdered Rhei Rhizoma (Daiou) and Sennae Folium (Senna) were purchased from Kinokuniya, an oriental crude drug store in Akihabara, Tokyo. The authentic samples of sennosides A and B were obtained from Funakoshi Chemical Co. All the other chemicals were of analytical grade.

Assay of the Inhibitory Effects The enzyme-inhibitory effects of the extracts, fractions and isolated components from both crude drugs were examined by means of the colorimetric method employing Determiner MAO, a clinical laboratory test kit for determination of human serum MAO activity. The procedures were previously reported (substrate, allylamine; standard enzyme, lyophilized bovine serum with known MAO activity; samples, the extracts, etc. dissolved in DMSO or 0.5% (v/v) aqueous $NaHCO_3$; inhibition rate, percent decrease in the absorbance at 660 nm).[26]

Extraction and Purification of Enzyme-Inhibitory Components Five hundred grams of powdered Rhei Rhizoma and 200 g of powdered Sennae Folium were extracted with 4 and 1.5 l respectively of 60% (v/v) MeOH at 50 °C for 2 h. After removing the residues by filtration, the filtrates were concentrated *in vacuo* below 50 °C. The combined concentrates were acidified to pH 1.5—2.0 by adding HCl, and then shaken in a separatory funnel with *n*-butanol (*n*-BuOH), until the *n*-BuOH layers showed no reddish color. The combined *n*-BuOH layers were dried overnight on anhydrous Na_2SO_4 followed by evaporation to dryness *in vacuo* below 50 °C. The residues thus obtained (the crude extracts) were treated with a small volume of cold MeOH. The procedures for isolation of enzyme-inhibitory components, I—IV, from the cold MeOH solutions of crude extracts, as well as the yields and inhibitory activities, are illustrated in Chart 1.

I (Sennoside A) and II (Sennoside B) The enzyme-inhibitory components, I and II, were recrystallized from 70% (v/v) aqueous acetone as yellow plates of mp 217—240 °C (dec.) and as yellow prisms of mp 205—212 °C, respectively. Compounds I and II were identical with authentic samples of sennosides A and B, respectively on the basis of melting points, spectral (UV, IR and ^{1}H-NMR) comparisons and chromatographies (TLC and PC). UV λ_{max} (in 0.5% aqueous $NaHCO_3$): I, 269, 308, 345 nm; II, 269, 308, 349 nm. IR (KBr): I, 3400, 2900, 1720, 1640, 1610, 1600, 1570, 1470, 1350, 1290, 1260, 1230, 1210, 1070, 900, 850 cm^{-1}; II, 3400, 2900, 1710, 1640, 1610, 1600, 1570, 1460, 1420, 1340, 1290, 1250, 1210, 1070, 900, 850, 760 cm^{-1}. ^{1}H-NMR (in DMSO-d_6): I, signals at 3.78—5.16 (sugar protons), 6.52 (10—10′) protons),[20] 7.24—7.72 ppm (aromatic protons); II, 3.68—5.02 (sugar protons), 7.28—7.50 ppm (aromatic protons). TLC (on Silica-gel G): I, Rf=0.35; II, Rf=0.22 using ethyl acetate (EA)–isopropanol–water (4:3:3, v/v) as the developing solvent. PC (on Whatman 3 MM): I, Rf=0.37; II, Rf=0.18 with *n*-BuOH–ethanol (EtOH)–0.2 M citrate buffer (pH 6.2) (2:1:2, v/v). The SI-MS spectral data of I (m/z=887 for $[M+H+Na]^+$) and II (m/z=886 for $[M+Na]^+$) ($M = C_{21}H_{19}O_{10}$) and the results of elemental analysis (%) (I: C, 55.91; H, 4.50; Ash, 3.20; II: C, 55.35; H, 4.41; Ash, 3.38) suggested that I and II were sennosides A and B, which were obtained as the sodium salts and/or

mixtures with inorganic materials, possibly due to the use of $NaHCO_3$ in the isolation procedures.

III (Sennoside E) and IV (Sennoside F) As in the cases of I and II, III and IV were recrystallized from 70% aqueous acetone as yellow needles of mp 209—214 °C and as a yellow powder of mp 167—173 °C, respectively. UV λ_{max} (in 0.5% aqueous $NaHCO_3$): III, 272, and 334 nm; IV, 270, and 328 nm. TLC (on Silica-gel G): both III and IV Rf=0.22 using EA–isopropanol–water (4:3:3, v/v) as the solvent. PC (on Whatman 3 MM): III, Rf=0.24; IV, Rf=0.11, with *n*-BuOH–EtOH–0.2 M citrate buffer (pH 6.2) (2:1:2, v/v). As described later, mild alkaline hydrolysis of III and IV gave I (in the case of III) and II (in the case of IV), in addition to oxalic acid, which was a common component from both III and IV. Thus, it was considered that III and IV were degraded, on mild alkaline hydrolysis, into I and oxalic acid, and into II and oxalic acid, respectively. The absorption maxima in the UV spectra of and Rf values in TLC and PC of III and IV agreed with those of sennosides E and F (oxalate esters of sennosides A and B), which were previously reported by Oshio *et al.*[20] as constituents of Rhei Rhizoma, respectively.

Mild Alkaline Hydrolysis of III and IV Followed by Identification of the Products About 10 mg of III and a small amount (less than 1 mg) of IV were left for 3 d in 10 ml of 10% and 0.5% aqueous $NaHCO_3$, respectively. Then, aliquous (2—20 μl) of the mixtures were injected into the isotachophoresis apparatus employing 0.01 M HCl (adjusted to pH 3.6 with β-alanine) and 0.01 M sodium caproate as leading and terminal electrolytes, respectively (the migration current was stabilized at 200 μA for 10 min, and at 100 μA thereafter). Oxalic acid (potential unit value: 0.11) was identified due to elongation of the appropriate zone in mixed charging with an authentic sample. On the other hand, the alkaline hydrolysates acidified by HCl were shaken with *n*-BuOH, and the *n*-BuOH layers were dried overnight on anhydrous Na_2SO_4, followed by evaporation to dryness *in vacuo* below 50 °C. The residues (deacylation products) were recrystallized from 70% (v/v) aqueous acetone, and identified as I (sennoside A) and II (sennoside B), respectively, in the same manner as already described. Thus, III and IV were sennosides E and F, oxalate esters of sennosides A and B, respectively.

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