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INHIBITION OF MOUSE LIVER SIALIDASE BY PLANT FLAVONOIDS

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Flavonoids (103 species) were tested for inhibitory activity against mouse liver sialidase using sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate (PNP-NeuAc) as substrate. Isoscutellarein-8-*O*-glucuronide from the leaf of *Scutellaria baicalensis* showed most potent activity (IC_{50}, 40 μM), and this flavone appeared to be a non-competitive inhibitor of the enzyme. This flavone inhibited the lysosomal solubilized sialidase against PNP-NeuAc and sialyllactose effectively, but not microsomal enzyme against gangliosides and colominic acid. whereas, negligible or weak inhibitory activities were observed for influenza virus sialidase, β-galactosidase, α-mannosidase, and α-glucosidase tested. These results indicate that this flavone may be useful to elucidate the function of the lysosomal solubilized sialidase.

Sialidase [neuraminidase, EC 3.2.1.18] catalyzes the removal of sialic acid residues from sialoglycoconjugates, and this removal is associated with the several important biological reactions (1,2), such as clearance of serum sialoglycoproteins, antigenic expression, and recognition by receptors. Sialidase activity has been studied in various kinds of mammalian tissues, and subcellular distribution, substrate specificity, and pH optimum of this enzyme have been reported (3,4), some of which have been purified recently (5-7). But the function of the sialidase *in vivo* is unclear. The specific inhibitor for mammalian

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Abbreviations: NeuAc2en, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; PNP-NeuAc; sodium *p*-nitrophenyl-*N*-acetyl-α-D-neuraminate.

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sialidase should be provided as useful tool to elucidate the function of the enzyme. The major flavones from the root of *Scutellaria baicalensis* were shown to have significant inhibitory activity against mouse liver sialidase (8). Therefore, the inhibitory activity of various plant flavonoids against this enzyme was tested with a new substrate for sialidase, PNP-NeuAc. The present paper describes the inhibitory activity of the flavonoids against mouse liver sialidase, and mode of inhibition by active flavonoid.

MATERIALS AND METHODS

Materials: Flavonoids were isolated or prepared as described previously (9-18). NeuAc2en was purchased from Boehringer Mannheim. PNP-NeuAc was a kind gift from Snow Brand Milk Products and Towa Kasei Kogyo, and was also purchased from Wako Pure Chemical Industries. Fetuin glycopeptides were prepared according to Spiro and Bhoyroo (19). Bovine brain gangliosides mixture and colominic acid were obtained from Iatron and Nacalai Tesque, respectively. G_{D3}, and $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$-sialyllactose were kind gifts from Snow Brand Milk Products. Mouse liver sialidase fractions were prepared as previously described (4). Influenza HA vaccine was obtained from the Kitasato Institute. Other PNP-glycopyranosides, jack bean α-mannosidase, Brewer's yeast α-glucosidase, and almond ß-glucosidase were purchased from Sigma. Jack bean ß-galactosidase was from Seikagaku Kogyo.

Sialidase Assay: Flavonoids were dissolved in 50% DMSO. Mouse liver sialidase activity was assayed as follows. The reaction mixture contained 25nmol of PNP-NeuAc, flavonoid solution (10 μl), and the enzyme in 0.21 ml of 12.5 mM citrate-phosphate buffer, pH 4.2. After incubation at 37°C for 2h, the reaction was stopped by the addition of 0.8 ml of ethanol, and allowed to stand at -20°C for 1h. Then the supernatant (0.15 ml) obtained by centrifugation was added to same volume of 0.2M sodium borate buffer, pH 9.8, in microtiter plate. The PNP liberated was determined from the absorbance at 405 nm with a Micro Plate Reader Model MPR-A4 (Tosoh). For the screening of sialidase inhibitor, 1,000 x *g* supernatant of mouse liver homogenate (4,8) was used as the enzyme. Influenza virus sialidase activity was assayed in 0.11 ml of 25 mM citrate-phosphate buffer, pH 5.0, containing 25nmol of PNP-NeuAc, flavonoid solution (10 μl), and influenza HA vaccine (10 μl) as the enzyme, then the reaction mixture was incubated at 37°C for 15 min in microtiter plate. The reaction was terminated with the addition of 0.19 ml of 0.2M sodium borate buffer, pH 9.8. Sialidase activity toward sialyllactose, colominic acid, and gangliosides were determined by thiobarbituric acid method (20) as previously described (4).

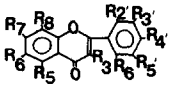
Other Methods: Jack bean α-mannosidase, jack bean ß-galactosidase, almond ß-glucosidase, Brewer's yeast α-glucosidase, and mouse liver lysosomal soluble ß-galactosidase activities were assayed in a similar manner as influenza virus sialidase, except for the reaction mixture containing 1.25 munit of glycosidase and 1mM appropriate PNP-glycopyranoside in 25mM sodium citrate buffer, pH 4.5, pH 3.5, pH 4.5, 25mM citrate-phosphate buffer, pH 6.8, or pH 3.6, respectively.

RESULTS AND DISCUSSION

A variety of flavonoids (80 kinds of flavones, 20 kinds of flavanones and 3 kinds of chalcones) were tested for inhibitory effect

on mouse liver sialidase activity. Four kinds of flavones (F3, F35, F46, and F57) inhibited more than 50% of the enzyme activity at final concentration of 48 μg/ml (Table I). Whereas influenza virus sialidase was inhibited weakly by these flavones even higher concentration of samples were used (Table II and data not shown). The flavanones (9-11,13,14,16-18) and chalcones (14,18) tested have little or no inhibitory activity against mouse liver enzyme (data not shown). These results indicate that flavone structure is necessary to inhibit mouse liver sialidase, and that inhibitors for mouse liver sialidase are different flavones from that for influenza virus enzyme. Previously, major flavones from the root of *S. baicalensis* were reported to have mouse liver sialidase inhibitory activity (8). In the present paper, wogonin (F17) showed significant inhibitory activity but baicalein (F5), baicalin (F10), and wogonin glucuronide (F22) showed no activity. These difference may be caused by the use of different substrate for mouse liver sialidase. The most potent inhibitor for mouse liver sialidase, isoscutellarein (5,7,8,4'-tetrahydroxyflavone)-8-*O*-glucuronide (F35), from the leaf of *S. baicalensis* (10) inhibited the enzyme in a dose-dependent manner (Fig. 1). This activity was similar with a known potent sialidase inhibitor, NeuAc2en (IC_{50}, 16 μM). Complete inhibition was observed with 103 μM of F35, and its IC_{50} value was estimated to be 40 μM. Mouse liver sialidase activity was measured at increasing PNP-NeuAc concentration in the presence or absence of 51 μM of F35. As seen in Fig. 1 inset, F35 decreased the maximal velocity of mouse liver sialidase three fold (0.66 to 0.22 μM/min) but did not significantly alter the Km value (0.26 mM) of the enzyme for PNP-NeuAc. Therefore, F35 appeared to be acting as a non-competitive inhibitor of mouse liver sialidase. The Ki value of F35 in this assay was 26 μM. This inhibition constant was calculated using the values of V_{max} obtained at 0 and 51 μM of F35. We have reported that mouse liver contains at least two kinds of sialidase, one of which is easily solubilized from lysosome

Table I. Inhibitory activity of flavones on mouse liver sialidase



No.	Substituent										Inhibition (%)	Reference and note
	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$		
F1	H	OH	H	OH	H	H	H	H	H	H	0	9
F2	H	OH	H	OGlcA	H	H	H	H	H	H	0	10
F3	H	OiPr	H	OiPr	H	H	H	H	H	H	51.2	11
F4	H	OH	H	OBz	H	H	H	H	H	H	48.0	11
F5	H	OH	OH	OH	H	H	H	H	H	H	0	11
F6	H	OAc	OAc	OAc	H	H	H	H	H	H	30.3	a
F7	H	OH	OMe	OH	H	H	H	H	H	H	0	11
F8	H	OH	OMe	OAc	H	H	H	H	H	H	0	b
F9	H	OH	OMe	OMe	H	H	H	H	H	H	7.0	12
F10	H	OH	OH	OGlcA	H	H	H	H	H	H	0	11
F11	H	OH	OH	OGlcAMe	H	H	H	H	H	H	0	11
F12	H	OH	OH	OGlc	H	H	H	H	H	H	3.5	11
F13	H	OH	OMe	OGlcA	H	H	H	H	H	H	0	11
F14	H	OH	OMe	OGlcAMe	H	H	H	H	H	H	8.1	11
F15	H	OH	OH	Ogent	H	H	H	H	H	H	0	12
F16	H	OH	H	OH	OH	H	H	H	H	H	1.5	11
F17	H	OH	H	OH	OMe	H	H	H	H	H	13.6	11
F18	H	OH	H	OBz	OH	H	H	H	H	H	10.3	11
F19	H	OH	H	OMe	OMe	H	H	H	H	H	12.5	11
F20	H	OMe	H	OH	OMe	H	H	H	H	H	13.3	11
F21	H	OMe	H	OMe	OMe	H	H	H	H	H	14.6	11
F22	H	OH	H	OGlcA	OMe	H	H	H	H	H	0	11
F23	H	OMe	H	OGlcA	OMe	H	H	H	H	H	0	c
F24	H	OH	H	OAc	OMe	H	H	H	H	H	0	11
F25	H	OMe	H	OAc	OMe	H	H	H	H	H	0	11
F26	H	OH	H	OH	H	OH	H	H	H	H	0	11
F27	H	OH	H	OH	H	H	H	OH	H	H	17.9	11
F28	H	OH	H	OGlc-Rha	H	H	H	OH	H	H	0	11
F29	H	OH	H	OGlc-Rha	H	H	H	OMe	H	H	0	d
F30	OGal-Glc	OH	H	OH	H	H	H	OH	H	H	44.3	11
F31	H	OH	OH	OH	H	H	H	OH	H	H	0	10
F32	H	OH	OMe	OH	H	H	H	OH	H	H	0	9
F33	H	OH	OMe	OMe	H	H	H	OH	H	H	0.2	11
F34	H	OH	OMe	OMe	H	H	H	OMe	H	H	24.4	10
F35	H	OH	H	OH	OGlcA	H	H	OH	H	H	100.0	10
F36	H	OH	H	OH	OMe	H	H	OH	H	H	29.5	11
F37	H	OH	OMe	OMe	OH	H	H	H	H	H	0	11
F38	H	OGlc	H	OH	H	H	OH	OH	H	H	29.4	11
F39	H	OH	H	OGlc	H	H	OH	OH	H	H	0	11
F40	H	OH	H	OH	H	OH	OH	H	H	H	0	11
F41	H	OH	H	OH	OMe	OH	H	H	H	H	0	11
F42	H	OH	H	OMe	OMe	OH	H	H	H	H	0	11
F43	H	OH	H	OH	OMe	OMe	H	H	H	H	0	11
F44	H	OH	H	OGlcA	OMe	OMe	H	H	H	H	0	13
F45	H	OH	H	OMe	OMe	OAc	H	H	H	H	0	11
F46	H	OMe	H	OMe	OMe	OMe	H	H	H	H	63.5	14
F47	H	OAc	H	OMe	OMe	OAc	H	H	H	H	0	11
F48	H	OH	OMe	OMe	OMe	OH	H	H	H	H	24.8	11
F49	H	OH	OMe	OH	H	H	OH	OH	H	H	0	e
F50	H	OH	OMe	OMe	H	H	OMe	OH	H	H	0	9
F51	H	OH	H	OH	OMe	OH	H	H	H	OMe	0	11
F52	H	OH	H	OH	OMe	OMe	H	H	H	OMe	8.6	14
F53	H	OH	H	OMe	OMe	OH	H	H	H	OMe	20.0	13
F54	H	OH	H	OMe	OMe	OMe	H	H	H	OMe	0	14
F55	H	OH	H	OMe	OMe	OMe	H	H	H	OBz	23.2	11
F56	OH	OH	H	OH	H	OH	H	H	H	OH	0	11
F57	OMe	OH	H	OMe	H	H	OMe	OMe	H	H	54.0	11
F58	ORha	OH	H	OH	H	H	OH	OH	H	H	0	11
F59	H	OH	OMe	OMe	OMe	OH	H	H	H	OMe	0	11
F60	H	OH	OMe	OMe	OMe	OMe	H	H	H	OMe	31.7	11
F61	H	OH	H	OH	OMe	OH	H	H	OH	OMe	0	11
F62	H	OH	Glc	OH	H	H	H	OH	H	H	19.7	11
F63	H	OH	Glc	OMe	H	H	H	OH	H	H	0	11
F64	H	OH	Glc	OGlc	H	H	H	OH	H	H	47.0	11
F65	H	OH	Glc	OMe	H	H	H	OMe	H	H	10.6	11
F66	H	OMe	Glc	OMe	H	H	H	OMe	H	H	24.2	11
F67	H	OAc	$GlcAc_4$	OMe	H	H	H	OAc	H	H	0	11
F68	H	OH	Glc	OMe	H	H	H	OGlc	H	H	11.4	15
F69	H	OH	Glc	OH	H	H	OH	OH	H	H	0	11
F70	H	OH	Glc	OMe	H	H	OH	OH	H	H	0	11
F71	H	OH	Glc-Rha	OMe	H	H	H	OMe	H	H	0	11
F72	H	OH	H	OH	Glc	H	H	OH	H	H	0	11
F73	H	OH	H	OMe	Glc	H	H	OH	H	H	0	11
F74	H	OH	H	OMe	Glc	H	H	OMe	H	H	0	11
F75	H	OMe	H	OMe	Glc	H	H	OMe	H	H	28.7	11
F76	H	OH	H	OMe	C_5H_9	H	H	OH	H	H	0	f
F77	OH	OH	H	OH	C_5H_9	H	H	OMe	H	H	0	11
F78	ORha	OH	H	OGlc	C_5H_9	H	H	OH	H	H	0	11
F79	ORha	OH	H	OGlc	C_5H_9	H	H	OMe	H	H	15.0	11
F80	OH	OH	H	$-OC(CH_3)_2(CH_2)_2-$		H	H	OMe	H	H	10.4	11

Abbreviations: Ac, acetyl; Bz, benzyl; C_5H_9, prenyl group; Gal, galactose; gent, gentiobiose; Glc, glucose; GlcA, glucuronic acid; iPr, isopropyl; Rha, rhamnose. Concentration of flavones; 48 μg/ml.

[a]prepared by acetylation of F5. [b]prepared by partial acetylation of F7. [c]isolated from *Scutellaria rivularis* (unpublished). [d]isolated from *Exacum walkeri* (unpublished). [e]isolated from *Helenium autumnale* (unpublished). [f]isolated from *Dolabella auricularia* (unpublished).

Table II. Inhibitory activity of F35 against various glycosidases

Glycosidase	Concentration (μM)	Inhibition (%)
Sialidase (influenza virus)	197	23.6
β-Galactosidase (mouse liver lysosomal solubilized)	51	7.4
β-Galactosidase (jack bean)	51	3.3
α-Mannosidase (jack bean)	51	1.7
α-Glucosidase (yeast)	51	2.2
β-Glucosidase (almond)	51	37.1

by hypotonic disruption, and another is bound to membrane, such as microsomal enzyme (4). The lysosomal solubilized sialidase is active toward oligosaccharides and glycopeptides, whereas microsomal sialidase is preferentially active to gangliosides and colominic acid (4). The relationship between structure of flavones and sialidase inhibitory activity was not clear (Table I). This result may be caused by the use

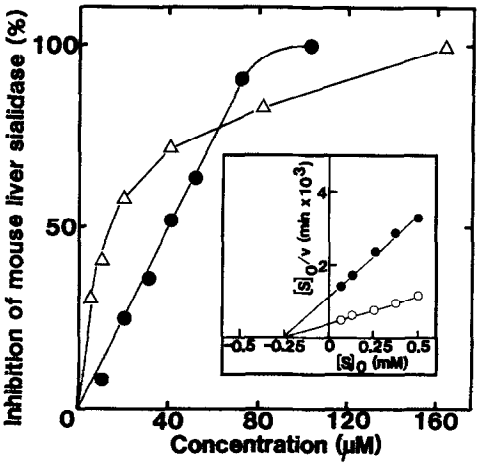


Fig. 1 Inhibitory activity of isoscutellarein-8-*O*-glucuronide (F35) against mouse liver sialidase. Sialidase inhibitory activities of F35 (●) was compared with that of NeuAc2en (△) under the condition described in MATERIALS AND METHODS. The inset shows $[S]_0/v$-$[S]_0$ plots of mouse liver sialidase for PNP-NeuAc. Mouse liver sialidase activity was assayed in the absence (○) or presence of F35 at 51 μM (●) in increasing substrate concentration.

Table III. Inhibitory activity of F35 against mouse liver sialidase fractions

Fraction	Substrate	Inhibition (%)[a]
Lysosomal solubilized	PNP-NeuAc	100
	α(2→3)sialyllactose	78.0
	α(2→6)sialyllactose	100
	Fetuin glycopeptide	0
Microsomal	Gangliosides mixture	0
	G_{D3}	14.1
	Colominic acid	15.4

[a] Concentration of F35; 51 μM.

of crude sialidase for the assay. F35 inhibited lysosomal solubilized sialidase activity toward PNP-NeuAc, and α(2→3) and α(2→6)sialyllactose, but little inhibited microsomal sialidase toward G_{D3} and colominic acid (Table III). F35 could not inhibited lysosomal solubilized sialidase toward fetuin glycopeptides. F35 also little inhibited other glycosidases, such as mouse liver lysosomal solubilized ß-galactosidase, jack bean ß-galactosidase, jack bean α-mannosidase, and yeast α-glucosidase, except almond ß-glucosidase when 51 μM of F35 was used (Table II). It has been reported that NeuAc2en inhibits mammalian sialidases against not only sialyllactose but also gangliosides (21,22) and *N*-(4-nitrophenyl)oxamic acid inhibits the enzyme against glycoproteins but not sialyllactose (21). These results indicate that F35 is potent and specific inhibitor for mouse liver lysosomal solubilized sialidase toward PNP-NeuAc and sialyllactose, and has distinct specificity from known sialidase inhibitors. The biological function of mammalian sialidase has not been well known. F35 should prove to be a useful specific inhibitor for mouse liver lysosomal solubilized sialidase in order to elucidate the biological function of the enzyme, which is predominant sialidase in mouse liver (4).

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