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EFFECTS OF FLAVONOIDS IN HYBRID MEMBRANES ON THE ACTIVITY OF TRIPEPTIDE AS AN ENZYME MODEL AND THEIR INHIBITION OF HYBRIDOMA GROWTH *IN VITRO*

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The enantioselective hydrolysis of amino acid esters catalyzed by the active tripeptide was enhanced by adding quercetin or quercitrin in the hybrid membranes. In toxicity tests with the human lymphoma-human lymphocyte B hybridoma *in vitro*, the viability and cell number growth in the hybridoma cells were greatly suppressed by the hybrid membranes with quercetin or quercitrin.

KEYWORDS —— enantioselectivity; amino acid ester; hydrolysis; hybrid membrane; flavonoid; hybridoma cell; *in vitro* antitumor activity

The functional molecular assemblies composed of surfactants and active peptides have attracted the attention of many researchers in Biomimetic Chemistry as one of the efficient enzyme models for clarifying the catalytic specificity of native enzymes.[1,2] In the organized hybrid assemblies of micellar and vesicular surfactants including the active tripeptide, high or perfect enantioselective catalysis for the discrimination of the L-form substrate from the D-form was attained by changing the ionic strength,[3] the species of surfactant,[4] and the composition of coaggregates.[5]

Also, plant flavonoids reveal many aspects that are of interest chemically and biochemically.[6] Especially, the effects of flavonoids on the activity of enzymes and inhibition of tumor promotion have been attracting considerable attention.[7] However, there has been no report of the effect of flavonoids as one of the important regulatory materials, such as cholesterol and unsaturated fatty acids, on stereoselective hydrolysis in molecular assembly systems.

In this paper, we report the effect of flavonoids on the enantioselective hydrolysis in the hybrid membranes of the double-chain and single-chain surfactants. We also investigated the cell toxicity of hybrid assemblies with and without flavonoids in the hybridoma cells *in vitro*.[8]

First, we examined the hydrolytic cleavage of long-chain enantiomers (p-nitrophenyl N-dodecanoyl-D(L)-phenylalaninates, D(L)-S_{12}) by the active tripeptide (N-benzyloxycarbonyl-L-phenylalanyl-L-histidyl-L-leucine, Z-PheHisLeu) with or without flavonoids in coaggregate systems composed of 59 mol% ditetradecyldimethylammonium bromide ($2C_{14}Br$) vesicles and 41 mol% hexadecyltrimethylammonium bromide (CTAB) micelles[9] in 0.2 M Tris buffer (μ = 0.2 with KCl) at pH 7.6 and

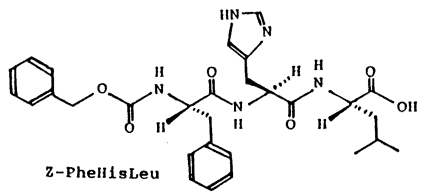
25°C. The clear stock solutions for kinetics were prepared by dissolving catalyst, surfactants, and flavonoids in Tris-KCl buffer with sonication (BranSonic ModelB 3200 apparatus, 90 W) at 50°C for 1 h.

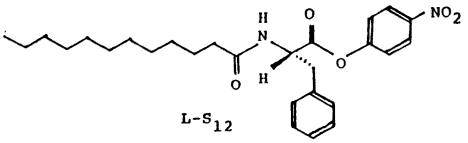
The results are summarized in Table I. The noteworthy aspects are: (a) The rate constants (k_s) for the hydrolytic cleavage of enantiomers (D(L)-S_{12}) without the catalyst (Z-PheHisLeu) were greatly elevated with the addition of quercetin, quercitrin, or rutin compared to the absence of flavonoids. Such an elevation was not observed in the presence of baicalein or baicalin. (b) The enantioselectivity (k_ψ^L/k_ψ^D) for the hydrolysis of D(L)-S_{12} as catalyzed by Z-PheHisLeu was enhanced in the presence of quercetin or quercitrin along with the suppression of the rate in the D-isomer substrate. But the k_ψ^L/k_ψ^D value in the presence of rutin

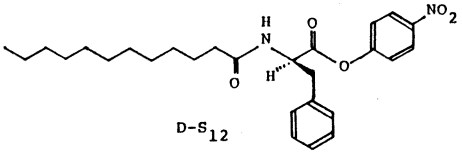
Table I. Rate Constants and Enantioselectivity for the Hydrolysis of D(L)-S_{12} with Flavonoids in the Presence of the Z-PheHisLeu Catalyst in the Hybrid Assemblies Composed of $2C_{14}Br$ and CTAB Surfactants[a)]

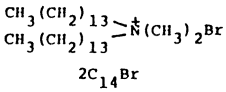
Flavonoid	k_s, s^{-1}		k_ψ, s^{-1}		L/D
	L	D	L	D	
No Additive	0.0081	0.0081	0.268	0.0117	23
Quercetin	0.0581	0.0581	0.278	0.0073	38
Quercitrin	0.0708	0.0844	0.187	≃ 0	
Rutin	0.0354	0.0354	0.199	0.0132	15
Baicalein	0.0105	0.0105	0.235	0.0102	23
Baicalin	0.0106	0.0115	0.270	0.0131	21

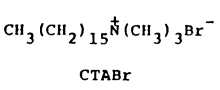
a) pH 7.6, 25°C, 0.2 M Tris buffer, μ = 0.2, 3% (v/v) CH_3CN-H_2O, [substrate] = 1×10^{-5} M, [Z-PheHisLeu] = 2×10^{-4} M, [$2C_{14}Br$] = 1×10^{-3} M, [CTAB] = 7×10^{-4} M. Rates of p-nitrophenol liberation from p-nitrophenyl esters were measured at 400 nm with a Hitachi 150-20 UV spectrophotometer. The reaction obeyed the usual pseudo-first-order rate law over 80% conversion, and the k_ψ value was evaluated from (k_t - k_s), where k_t and k_s denote the first-order rate constants with and without catalyst, respectively. The rate constants are reproducible within ± 4.0%.

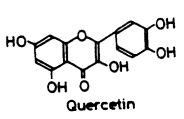


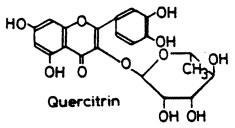


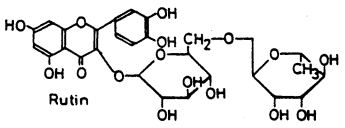


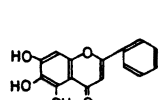




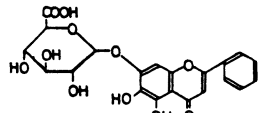








Baicalein



Baicalin

was reduced compared with that in the absence of flavonoid. No effect was obtained in the presence of baicalein or baicalin. It is deduced from these results that quercetin and quercitrin, commonly having two hydroxy groups at the 3' and 4' positions, may play an important role in the enhancement of spontaneous and enantioselective hydrolysis of the long-chain substrates in the hybrid membranes.

The lactate transport and glycolysis in Ehrlich ascites tumor cells were inhibited by quercetin.[10] Also, quercetin affected aerobic glycolysis and the growth of tumor cells.[11] So, we examined secondly the effect of quercetin and quercitrin, which enhanced the enantioselectivity, on their toxicity in hybridoma cells.[12] The toxicity experiments in vitro are summarized in Table II and the results are: (a) Quercitrin dissolved with dimethyl sulfoxide in aqueous solution slightly suppressed the viability of hybridoma cell. (b) There was strong toxicity in hybrid membranes composed of 59 mol% $2C_{14}Br$/41 mol% CTABr and 77 mol% $2C_{16}Cl$/23 mol% CTACl surfactants above the critical micelle concentration (cmc) of coaggregates.[13] (c) In the hybrid membranes composed of $2C_{16}Cl$ and CTACl, no viability was attained by adding quercetin and quercitrin reduced the cell number to half.

In conclusion, quercetin or quercitrin in the hybrid membranes enhanced the enantioselectivity for the hydrolysis of amino acid esters and suppressed the hybridoma growth *in vitro*. These results indicate that the suppression of the cell growth may be closely related to steric control in membrane proteins.

Table II. Toxicity of Hybrid Membranes Including Flavonoids in Hybridoma Cells[a)]

Additive	Cell number, $\times 10^4$ ml^{-1}	Viability, %
Quercetin[b)]	171 ± 6 (191)	70 (71)
Quercitrin[b)]	182 ± 12 (216)	69 (80)
$2C_{14}Br$ + CTABr[c)]	14 ± 3 (129 ± 17)	0 (88)
$2C_{16}Cl$ + CTACl	49 ± 4 (133 ± 12)	12.7 (95.4)
Quercetin + $2C_{16}Cl$ + CTACl	48 ± 7 (133 ± 12)	0 (95.4)
Quercitrin + $2C_{16}Cl$ + CTACl	27 ± 7 (133 ± 12)	16.4 (95.4)

Values in parentheses are those in the absence of additives.
a) 1×10^{-4} M phosphate buffer, [Flavonoid] = 3×10^{-6} M, [$2C_{14}Br$] = [$2C_{16}Cl$] = 2×10^{-5} M, [CTABr] = 1.4×10^{-5} M, [CTACl] = 6×10^{-6} M. Initial cell number: $(13-10)\times10^4$ ml^{-1}.
b) 0.01% dimethyl sulfoxide, [Flavonoid] = 2×10^{-6} M.
c) 2×10^{-4} M phosphate buffer.

$CH_3(CH_2)_{15}\overset{+}{N}(CH_3)_3Cl^-$ CTACl

$CH_3(CH_2)_{15}\overset{CH_3(CH_2)_{15}}{\underset{CH_3(CH_2)_{15}}{}}\overset{+}{N}(CH_3)_2Cl^-$ $2C_{16}Cl$

Toxicity experiments *in vivo* are now under consideration. The toxicity test of hybrid membranes including substances cancerocidal to tumor cells *in vitro* may be a promising screening method before being investigated *in vivo*.

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12) Cells were cultured for 4 days at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cell number was determined with a Cell Counter (Toa-Iryo CC-130 A). Viability was evaluated from $100C_a/(C_a + C_d)$, where C_a and C_d denote the alive and dead cell number, respectively. The dead cell number was counted with a hematometer after addition of trypan blue to the cell suspension.
13) The respective cmc values of $2C_{14}Br$/CTABr and $2C_{16}Cl$/CTACl coaggregates were estimated by the conductivity method to be 6.5×10^{-6} M (on the basis of $2C_{14}Br$) and 8.5×10^{-6} M (on the basis of $2C_{16}Cl$).

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