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Enzymatic Synthesis of Butyryl-Rutin Ester in Organic Solvents and Its Cytogenetic Effects in Mammalian Cells in Culture

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ABSTRACT

Enzymic acylation of a flavonoid, rutin, with trichloroethylbutyrate (TCEB) has been performed by subtilisin protease in anhydrous pyridine solution. The addition of a hydrophobic compound on rutin is expected to change the hydrophilic/hydrophobic balance of the molecule, giving new properties to this compound.

This work aimed at investigating the various cytological properties of the rutin-ester and compared them with those of the native molecule. No difference in the levels of sister chromosomes exchange (SCE) between rutin and rutin-ester treated cells at doses varying from 25 to 200 μg/mL was found. On the contrary impressive difference in the induced frequency of micronuclei (MN) between rutin and rutin ester treated cells was observed, for example, at a dose of 100 μg/mL of rutin were 3.5% MN counted, whereas for a similar dose treatment with rutin-ester a frequency of 8% of MN was found.

The fact that rutin-ester is causing significantly higher levels of MN than the rutin alone can be considered as a manifestation of a higher action of the agent on the chromosome owing to its easier penetration in to the cell after its esterification.

Index Entries: Enzymic catalysis in organic solvents; rutin; flavonoids; micronuclei; cytogenetic effects.

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INTRODUCTION

The importance of flavonoids lies in their widespread occurrence in human foods and in their use in more purified forms as drugs and food supplements. Humans consume daily about 1 g of flavonoids (*2*), a large part of which consists of the flavonol quercetin and its glycoside rutin (*11*). It is widely accepted that flavonoids possess antioxidant, antimicrobial, antimutagenic, anticarcinogenic and other properties (*2,8,10*). It is these characters, in combination with the presence in their molecules of carbohydrate moieties with multiple hydroxyl groups, that represent a particularly challenging target for regioselective modification, and render flavonoids an interesting field for biotransformations through enzymic procedures (*9,7,13*).

In the present work a form of rutin, acylated by a fatty acid using a protease, has been studied for its cytogenetic properties on V-79 cells in culture. The ester has been available through transesterification of the flavonoid with trichloroethylbutyrate (TCEB) catalyzed by subtilisin in an anhydrous pyridine solution. This enzymic esterification of rutin as well as the chemical characterization of the product have been described by Danieli et al. (*28*). The enzymic bioconversions in nonaqueous media is one of the main recent achievements of biotechnology. Many enzyme-catalyzed reactions practically impossible in water are readily carried out in organic solvents. This is due not only to a solubility factor and an equilibrium shift of the reaction, but also to novel catalytic properties and markedly enhanced operational stability displayed by enzymes in low water environments (*14,15,27*).

The esterification of the flavonol glycoside molecule by the addition of the fatty acid hydrophobic chain, is expected to change the hydrophilic/hydrophobic balance of the molecule, which may acquire novel properties. One of them could be a higher degree of penetration through the cell membrane, a characteristic quite common for amphiplilic molecules (*24*). This paper examines the activity of the enzymatically synthesized rutin-ester at the cytogenetic level and compares it with the activity of the native molecule.

MATERIALS AND METHODS

Materials

Protease from *Bacillus subtilis* (subtilisin EC3.4.21.14) was purchased from Sigma (St. Louis, MO) as lyophilized powder. Commercially obtained pyridine was purified and dried, rutin was purchased from Sigma, fetal calf serum was from Flow Labs (McLean, VA), 5-bromodeoxyuridine from Gibco Bio-Cult (Gaithersburg, MD), DMSO from BDH Chemicals Ltd.,

England, colchicine from Serva (Heidelberg, Germany) TLC plates were from Merck (Darmstadt, Germany). All other chemicals used in this work were obtained commercially and were of analytical grade. All solvents were of the highest purity commercially available and were used without further purification. When needed, solvents were dried by gentle shaking with 3A molecular sieves (Linde).

Methods

Preparation of Subtilisin

Prior to use the enzyme was dissolved in 0.1*M* phosphate buffer pH 7.8 and then it was lyophilized. This "pH adjustment" markedly increases catalytic activity of subtilisin in organic solvents (*14*).

Preparation of Trichlorethylbutyrate

Trichlorethylbutyrate (TCEB) was synthesized from butyryl chloride and 2,2,2-trichlorethanol following a general methodology (*25*).

Rutin-Ester Synthesis

The enzymatic transesterification reaction was followed by measuring the formation of the new ester by high-pressure liquid chromatography (HPLC). A μBondapak C18 125A, 10 μm (3.9 × 300 mm) Waters column with a Lambda Max model 481 LC spectrophotometer were used. A Metrohm Karl-Fischer titrator was used for water content measurements of the system.

In a typical procedure 0.3 mmol of rutin and 2.7 mmol of TCEB were placed in 1 mL of dry pyridine solution, whereas 100 mg of subtilisin were added and sonicated in order to reduce the size of the enzyme suspended molecules, since subtilisin is not dissolved in organic solvents. Then the suspension was placed in 45°C under vigorous stirring. Aliquots were withdrawn periodically, the enzyme was removed and the sample was subjected to thin layer chromatography (TLC), using precoated silica gel plates. The various samples were developed with the system: AcOEt; AcOH; H_2O 250; 1; 2. The product, which has an $Rf = 0.65$, was extracted from the plate, dissolved in methanol, filtered, and passed through the HPLC column described above. A 15 min linear gradient from 10 to 60% acetonitrile in water (containing 0.1% CH_3COOH) was employed. The flowrate was 1 mL/min and the detection was done at 254 nm.

Sister Chromatid Exchange (SCE) Studies

V-79 cells were grown in Eagles's minimal essential medium (modified) with Hank's salts and supplemented with 10% fetal calf serum. Two hours after the cells were seeded in 25 cm^2 culture flasks 5-bromodeoxyuridine at a concentration of 5 μg/mL and a rutin and rutin-ester dose, dissolved in a total volume of 0.025 mL of DMSO, were added to the cultures. All cultures were kept in complete darkness at 37°C in a 5% CO_2

atmosphere for a total of 26 h, with $10^{-6}M$ colchicine having been added for the last 2 h. Mitotic cells were harvested by shaking, treated with hypotonic solution and fixed with ethanol acetic acid solution. Air-dried slide preparations were processed by the fluorescent-plus Giemsa (FPG) technique of Perry and Wolf (*22*) to demonstrate sister-chromatid differentiation and scored under the microscope. Twenty-five or more metaphases were scored for each treatment group.

Induction of Micronuclei

A previously described method was used (*1*). Briefly, 60 mm Petri dishes containing 2 sterile cover slips each, were seeded with 1×10^5 V-79 cells/plate, 16 h before treatment. After treatment with various doses of rutin and rutin-ester cells were incubated for 24 h. Slides with the attached cells were treated with 0.075*M* KCl for 10 min, fixed with methanol for 10 min, and stained (Giemsa/H_2O, 1/6, 13 min). One thousand cells were scored for each dose, taking into account the criteria for identifying micronuclei (*6*).

RESULTS AND DISCUSSION

Rutin Acylation by Subtilisin

The physiological role of *Bacillus subtilis* protease is the hydrolysis of water soluble proteins (*21*). Recently proteases have also been used for catalysis in nonaqueous solvents, replacing hydrolytic with synthetic activity and a number of important reactions as peptide synthesis, esterification of sugars, and related compounds, have been reported (*5,23,26,28*). In our studies we used *Bacillus subtilis* protease (subtilisin) to introduce a butyryl moiety to rutin, a flavonoid diglucoside. Rutin was used as the nucleophile in the transesterification reaction, and the second substrate of the reaction, butyric acid, was used in its active trichlorethyl ester form. The reaction was carried out as described in the Materials and Methods section and the product was obtained by HPLC. The chromatograms show that retention time of rutin was 12.3 min, whereas of the product was 15.62 min (Fig. 1). The water content of the system was calculated by the Karl-Fischer method and found 0.3% (v/v). After about 8 h of reaction almost 90% of rutin was converted to butyryl-rutin ester. No reaction was observed in the absence of the enzyme or if the enzyme was used inactivated. The esterification has been identified to occur exclusively on the sugar moiety of rutin (*13*). After completion of the reaction, the insoluble enzyme can be recovered by filtration or centrifugation and used again.

Genotoxic Activity Studies

Tables 1 and Fig. 2 indicate the results for SCE and micronuclei (MN) induction respectively, which is following the treatment of V-79 cells with

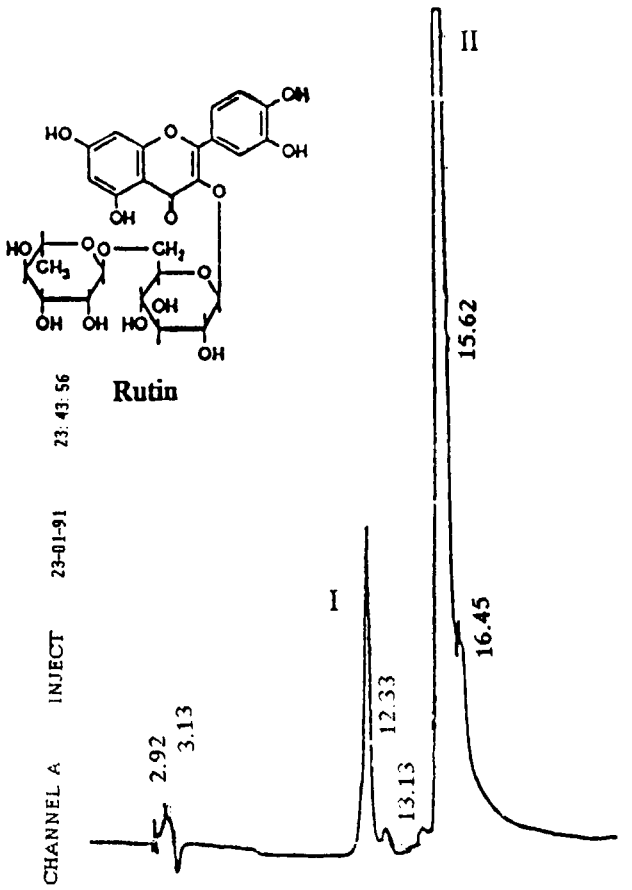

Fig. 1. The HPLC profile of rutin (1) and rutin ester (II), employing a 15-min linear gradient from 10 to 60% acetonitrile in water. The flowrate was 1 mL/min and the detection was done at 254 nm. The retention time for rutin is 12.3 and for the rutin-ester is 15.62 min.

Table 1
Effect of the Flavonoid, Rutin, and the Rutin-Ester on SCE in V-79 Cells

Agent	Doses, μg/mL	No of SCE metaphase Range	Mean ± SEM	*t* value comp. with	*p*
	0	2–8	6.12 ± 0.3		
	25	3–10	6.32 ± 0.35	0.48	>0.1
Rutin	50	5–12	7.52 ± 0.34	2.90	<0.1
	100	5–11	7.56 ± 0.32	3.04	<0.01
	200	4–12	7.76 ± 0.40	3.22	<0.01
	0	2–8	5.07 ± 0.33		
	25	3–10	5.76 ± 0.41	1.29	>0.1
Rutin-ester	50	3–9	5.92 ± 0.34	0.49	>0.1
	100	4–12	7.80 ± 0.41	5.11	<0.1
	200	4–13	7.38 ± 0.41	3.93	<0.1

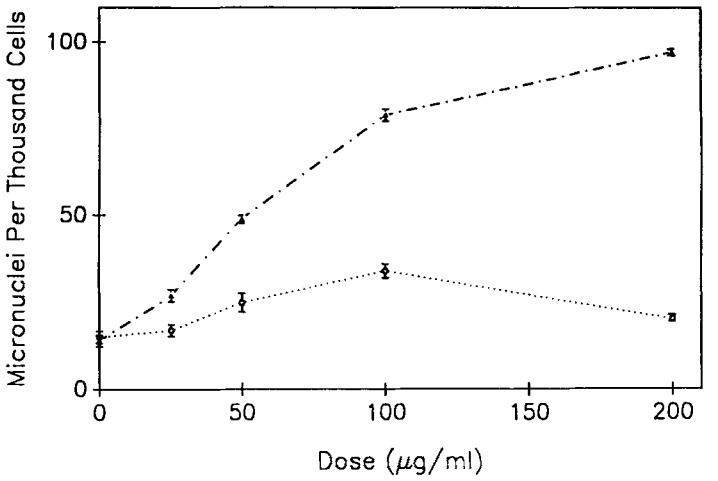

Fig. 2. Frequencies of micronuclei induced by the flavonoid glycoside rutin (o), and the rutin-ester (▲) on V-79 cells. Date represent mean values + SD from two independent experiments*. (*$P > 0.05$, between the two groups of data, for rutin and rutin-ester, estimated by the t-test).

various doses of the flavonoid rutin, as such, or when the flavonol glycoside molecule was acylated with the hydrophobic fatty acid chain. Both, the rutin and the rutin-ester induced insignificant increases of SCE. No significant differences in SCE induction were observed between rutin and rutin-ester. However, significant differences in the action of the two compounds was observed in the induction of micronuclei (MN). Both of them induced significantly higher levels of MN, but compared to the controls, the levels of MN that the rutin-ester molecule induced were two or three times higher than the ones caused to the same dose of rutin.

The ability of the quercetin glycoside rutin to induce genetic damage in mammalian cells has previously been reported (*16,17*) and the observed responses appear to be consistent with a mechanism of damage leading predominantly to DNA strand breaks, rather than the specific locus mutation (*18,19*). This is in agreement with the observation that both rutin and its ester, during the present work, were found to induce significant increases of micronuclei in V-79 cells, whereas SCE, a phenomenon known to be related to the point-mutation type of mutagenicity (*3*) remained low.

Data on the clastogenicity of this group of flavonoids are available for quercetin, which has been found to induce chromosomal anomalies in cultured cells, whereas conflicting results were obtained in the micronucleus test in vivo (*4*). The fact that quercetin has been found to be carcinogenic in experimental animals (*12*) imposes the use of its noncarcinogenic glycoside derivative, rutin, for pharmaceutical, commercial, or any other use necessary. However, under the condition that this glycoside derivative is not lacking also genotoxic properties, since, it induces micronuclei in mammalian cells, renders its application for human purposes in low quantities a necessary task.

An interesting contribution for this type of approach is derived from the results of the micronucleus test with the rutin-ester. As it is indicated in Fig. 2, for the same range of concentrations, rutin ester induced significantly higher levels of MN compared to rutin. It is suggested that this increased activity of the rutin ester is not a result of an increased toxicity of the molecule but rather to a higher degree of penetration through the living cell membrane of the esterified glycoside. This is not surprising but rather an expected property of this ester molecule, since the addition of a hydrophobic chain to the rutin molecule is expected to change the hydrophilic/hydrophobic balance, giving new properties to the compound. One of these can be the increased ability of penetration through the cell membrane. On the other hand, flavonol glycosides are usually nonmutagenic, unless the sugar moiety has been removed by a mixture of hydrolyzing glycosidases (*20*). The enzymatic addition of the acyl moiety to flavonoids has been reported to occur on C-3 of the sugar moiety (*7*). So, we can assume that the esterification of rutin does not give to the molecule any characteristic to make it more accessible to hydrolysing glycosidases. Thus, the increased penetration hypothesis seems to be the most reasonable one. We can conclude that in the case of the enzymatic modification of flavonoids in nonaqueous media, we are dealing with a biotechnological procedure that might hopefully allow the application of smaller quantities of certain flavonoids that will have a high degree of efficiency in their action on various biological procedures.

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