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Enzyme-Mediated Regioselective Acylations of Flavonoid Glycosides

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Abstract: *Flavonoid glycosides, xanthorhamnins B, C, and rutin have been acylated by the catalytic action of the protease subtilisin in anhydrous pyridine. The acylation occured with high yield with rutin giving a single monoester on its glucose moiety showing excellent selectivity. But it occured with low yield on the galactose moiety of the two flavonoid triglycosides.*

Key words : *Acylated flavonoid glycosides, enzymatic acylation.*

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Flavonoit Glikozitlerinin Enzimatik Açillenmesi

Özet: *Flavonoit glikozitlerinden ksantorannin B, C ve rutin, anhidr piridinde proteaz subtilisin ile açillenmiştir. Reaksiyon sonucunda, glukoz üzerinden rutinin monoesteri yüksek verimle elde edilirken, galaktoz üzerinden flavonoit triglikozitlerinin esterleri çok düşük verimle elde edilmiştir.*

Anahtar kelimeler : *Ester flavonoit glikozitler, enzimatik açilleme*

Introduction

Flavonoid glycosides are widely distributed in nature and often found as esters with different acids at specific positions of their sugar moieties. Besides these esters, the cinnamoyl, p-coumaroyl and feruloyl derivatives are the most frequent ones, some of which have pharmacological activities, e. g. the major compounds of the extract from *Ginkgo biloba* are the p-coumaroyl derivatives of quercetin and kaempferol glucorhamnosides. These esters are believed to have effects on the symptoms of cerebrovascular insufficiency and poor arterial circulation displayed by the extract [1,2].

These acylglycosides cannot be obtained by direct chemical esterification, thus an enzyme-mediated approach to the derivatives would be of particular interest. In recent years the proteolytic enzyme subtilisin has been used in organic solvents to catalyze the regioselective acylation of polyhydroxylated compounds[3,4].

We now report on the substilisin-catalyzed esterification of two flavonoid triglycosides isolated from *Rhamnus petiolaris* with high yield and commercial rutin, which has the diglycosidic moiety rutinose.

Material and Methods

General procedures: 1H-NMR spectra were recorded on Bruker AM 400 and Bruker WM 300. ESI-MS (Electro-Spray Ionisation Mass Spectrum) was recorded on Finnigan TSQ-700. Enzymatic transesterifications were followed by HPLC: HP (Hewlett Packard; HP 1040M Diode Array detector, reading at 254 and 350 nm; Nucleosil 100 5 μC_{18}; isocratic 5 % HCOOH/MeOH 40:60; flow rate 1 mL/min. The acylated compounds were identified by their higher retention times and unchanged chromophores. An Eppendorf Thermomixer 5436 was used throughout the study as incubator. For the distillation of synthetic TFEB and TFEC, a Büchi GKR-51 glass tube oven was used.

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Materials:

Xanthorhamnins B and C: Dried fruits of *Rhamnus petiolaris* Boiss. were extracted with several solvents and two major flavonoid triglycosides were isolated and purified by chromatographic methods. Their structures were identified by spectral methods [5].

Subtilisin (EC 3.4.21.14, protease from *Bacillus licheniformis*) was obtained from Sigma. It was dissolved in H_2O and the solution adjusted to pH 7.8 and freeze dried.

Rutin was from Aldrich.

Pyridine (anal. grade) was used without further purification, apart from drying by shaking with 3-Å molecular sieve (Merck).

Trifluoroethyl butanoate(TFEB) was synthesized from butyryl chloride and 2,2,2-trifluoroethanol in the presence of N,N-dimethyl-4-pyridinamine by general methodology[6]. It was purified by distillation at 106° and tested by NMR spectroscopy. The following characteristics had been obtained: ^{1}H-NMR (80 MHz, $CDCl_3$): δ 4.46 (q, J = 8.4 Hz), 2.39 (t, J= 7.5 Hz), 1.69 (m, J=7.5 Hz), 0.97 (t, J=7.5 Hz). These results were in good agreement with the reported data for TFEB[7].

Trifluoroethyl cinnamate(TFEC) was synthesized from cinnamoylchloride and 2,2,2-trifluoroethanol in the same way as TFEB. It was tested by NMR spectroscopy and the following characteristics had been obtained: ^{1}H-NMR (80 MHz, $CDCl_3$) : δ 4.61 (q, J = 8.6 Hz), 6.49 (d, J = 16 Hz), 7.81 (d, J = 16 Hz), 7.55 - 7.36 (5xArom. H).

Enzymatic acylations of rutin and xanthorhamnins: Subtilisin (35 mg) was added to 1 mL of anhydrous pyridine containing 50 µL substrate (30 mg Rutin, 40 mg Xanthorhamnin B and C), 30 µL trifluoroethyl butanoate and 40 µL trifluoroethyl cinnamate. The suspensions were shaken at 45° with 1400 rpm. After 2 days, RuBu, XCBu, XBBu were formed in 20.8 %, 0.7 % and 1.5 % yield, respectively. On the 5th day, RuBu was obtained with 23 % yield. Next day 50 µL TFEB was added again. On the 8th day, RuBu, XCBu and XBBu were observed with 36 %, 2 % and 2.9 % yield respectively. On the same day, 75 µL TFEB and 100 mg TFEC was added. After 12 days from the beginning, RuBu was obtained with 61 % (Figure 1)

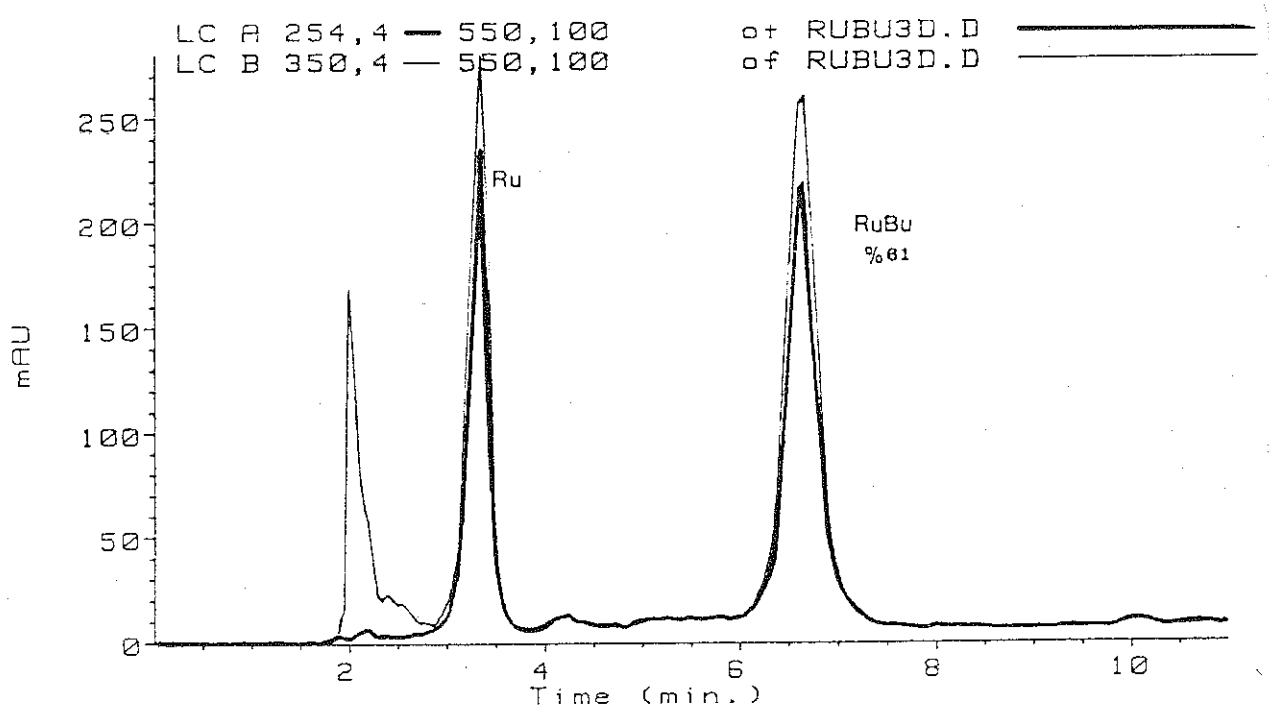


Figure 1. The yield of RuBu established by HPLC on the 12th day.

and RuCi was obtained with 3.6/1.8 % yield. However, XCCi and XBCi couldn't be obtained. On the same day, 100 µL TFEB was added again. RuBu was formed in 84 % yield at the end of two weeks. The enzyme was removed by filtration, the solvent evaporated and the crude residue purified by silica gel chromatography ($CHCl_3$: MeOH: H_2O; 80:20:2 as the solvent).

Results and Discussion

Lipases can catalyze the enzymatic acylation of primary hydroxyl groups in various unprotected monoglycosides, but only *Porcine pancreatic* lipase and *Chromobacterium viscosum* lipase are active in pyridine. Porcine pancreatic lipase, which regioselectively acylates the primary hydroxyl group of monoglycosides in pyridine, was found to be unreactive with di- and oligoglycosides[8].

Enzymatic acylation of sugars in water is thermodynamically inconvenient and therefore expensive cofactors are required as a source of free energy. Before the process of acylation, pyridine, which is one of a few organic solvents capable of dissolving sugars and the enzyme, are dried to eliminate hydrolysis of 2,2,2-trifluoroethyl butanoate. In the case of hydrolysis, the enzymatic acylations are not possible in water[7,8,9].

The proteolytic enzyme subtilisin is both stable and active in numerous anhdyrous organic solvents including pyridine. It can regioselectively acylate di- and oligoglycosides, nucleosides and related large molecules[10]. In several studies, subtilisin was used to introduce a butyryl moiety into carbohydrates, e.g., the acylation with subtilisin occurs at OH-C (6") or OH-C (3") of the glucose moiety. If OH-C (6") is blocked in the intersugar linkage, then the selectivity for OH-C (3") is expected. In addition, selectivity is independent of the presence and nature of the aglycone. It has been shown that the presence of a large aglycone moiety doesn't significantly reduce the reactivity of the substrate. In another example, the enzymatic butanoylation of the rhamnoglucoside naringin, in which the interglycosidic linkage is between C (1'"), of rhamnose and C (2") of glucose, occurred as 6"-0-butanoyl ester with subtilisin as expected. On the other hand, when rhamnose was replaced by another sugar like arabinose, the estirification occured on the arabinose moiety in addition to glucose[3]. This shows that subtilisin cannot acylate the rhamnose unit.

As subtilisin was found to be favourable for acylations of glycosides in previous studies, we preferred to use this enzyme in our study.

The two flavonoid triglycosides (named as xanthorhamnins) used in our investigation have the structures as rhamnazin 3-0-[0-α-L-rhamnopyranosyl-(1→3)-0-α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside (rhamnazin-3-0-β-rhamninoside= xanthorhamnin C) and rhamnetin-3-0-[0-α-L-rhamnopyranosyl-(1→3)-0-α-L-rhamnopyranosyl-(1→6)] - β-D-galactopyranoside (rhamnetin-3-0-β-rhamninoside = xanthorhamnin B).

The third compound rutin, has the diglycosidic moiety rutinose[6-0-(α-L-rhamnopyranosyl)-D-glucose], which is linked to OH-C(3) of the quercetin aglycone. When a solution of rutin in anhydrous pyridine was treated at 45° with an excess of trifluoroethyl butanoate in the presence of subtilisin, 84 % conversion was observed after two weeks. In a previous study, 65 % conversion was observed after 48 h, with the same agents, under the same conditions [3].

This shows that the yield of product increases depending on time. In our study, TFEB was added in five portions instead of adding the whole amount at once, as stated in the previous study[3]. This is another factor that effects the percentage of the conversion, as well as duration. During the acylation of rutin, the selectivity for OH-C (3") of glucose was expected, since OH-C (6") is blocked in the intersugar linkage. As a result of the reaction, a single product was

Abbreviations

RuBu : Rutinbutyrate
RuCi : Rutincinnamate
XBBu : Xanthorhamnin B butyrate
XCBu : Xanthorhamnin C butyrate
XBCi : Xanthorhamnin B cinnamate
XCCi : Xanthorhamnin C cinnamate
TFEB : Trifluoroethyl butanoate
TFEC : Trifluoroethyl cinnamate

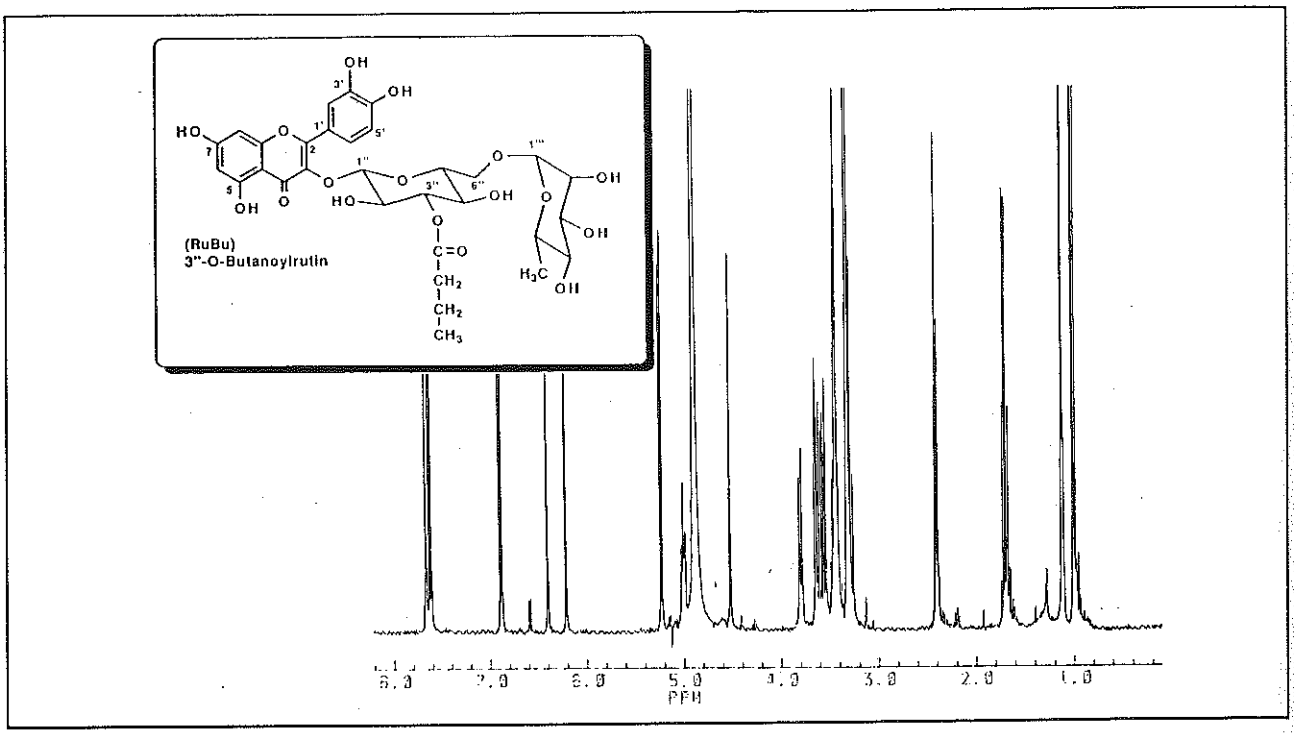


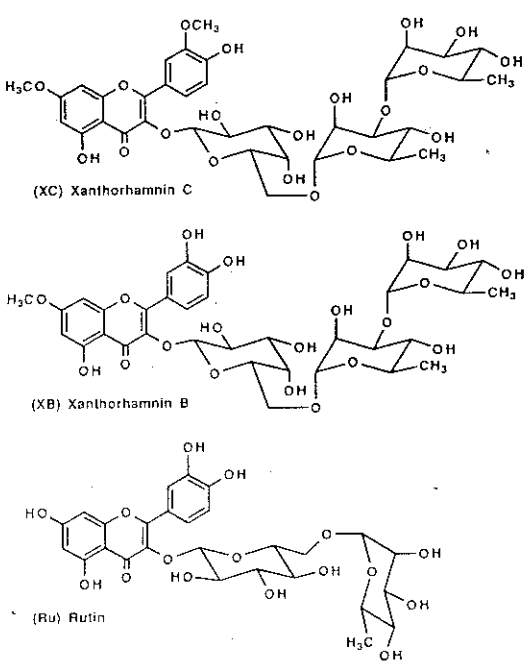
Figure 2. ^{1}H-NMR Spectrum of 3''-0-Butanoylrutin (RuBu) (300 MHz, MeOH-d_4)

Table 1. ^{1}H-NMR Spectral Data for Rutin (Ru) and 3''-0-Butanoylrutin (RuBu)

* 200 MHz, in DMSO-d_6

** 300 MHz, in MeOH-d_4

	H	RU* δ(ppm)	RUBU** δ(ppm)	J(Hz)
Aglycone	6	6.2d	6.20 d	(2.0)
	8	6.3 d	6.39 d	(2.0)
	2'	7.5 d	7.65 d	(2.2)
	5'	6.8 d	6.87 d	(8.5)
	6'	7.5 dd	7.61 dd	(2.2/8.5)
Glucose	1''	5.3 d	5.22 dd	(7.9)
	2''	3.0 - 3.7	3.60 dd	(7.9/9.5)
	3''	3.0-3.7	5.00 t	(9.2)
	4''	3.0-3.7	3.4-3.5	
	5''	3.0-37	3.4-3.5	
	6''	3.0-3.7	3.79 br d	(9.7)
Rhamnose	1'''	4.3 d	4.51 d	(1.6)
	2'''	3.0-3.7	3.64 dd	(1.6/3.4)
	3'''	3.0-3.7	3.54 dd	(3.4/9.5)
	4'''	30-3.7	3.27 t	(9.5)
	5'''	3.0-3.7	3.4-3.5	
	6'''	1.01 d	1.11 d	(6.2)
Butanoyl	CH_3-		0.99 t	(7.4)
	-CH_2-		1.69 m	
	-CH_2-CO		2.40 t	(7.4)



formed, which was isolated and purified by chromatographic methods and identified as 3"-0-butanoylrutin by spectroscopic properties(UV, IR, NMR, ESI-MS). On comparison with the ^{1}H-NMR spectra of rutin and rutinbutyrate (RuBu) (Figure 2) (Table 1) the signal corresponding to H-3" of glucose for RuBu was found downfield indicating the site of acylation. On the other hand, the ESI-MS exhibited a peak at m/z 704,3 $[M+H+Na]^+$ that supported the proposed structure.

However, the two flavonoid triglycosides had very low reactivities in the subtilisin catalyzed transesterification with trifluoroethyl butanoate under the same conditions(3 % after 8 days), possibly due to the presence of the galactose unit. From the results of this study and from those reported in previous communications, it has been shown that subtilisin is not a suitable enzyme for acylations of rhamnose and galactose moieties. Also it was obvious that the processes, which were made in this study to create cinnamic acylations, were not successful as stated in the previous study[4].

Based on the successful results of the rutin acylation, it is considered that the acylation of xanthorhamnins with suitable enzymes will be possible in further studies.

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