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Chemoenzymatic preparative separation of silybins A and B

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ABSTRACT

Robust and scalable chemoenzymatic procedure for obtaining gram amounts of pure silybin A and B within one week is described. It consists of the preparation of 23-*O*-β-pentaacetyl-galactopyranosides of silybin, their separation by silica gel chromatography, deprotection, and enzymatic sugar removal with β-galactosidase.

1. Introduction

The flavonolignan silybin (**1**) (CAS No. 22888-70-6) is the major component of the silymarin complex extracted from the seeds of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae) (milk thistle). Besides the main component silybin (ca. 30%) (Fig. 1), silymarin contains its congeners, such as isosilybin, silychristin, isosilychristin, silydianin, 2,3-dehydrosilybin, and taxifolin [1,2] and about 20–30% is an undefined polymeric phenolic fraction [3].

Silymarin flavonolignans are biogenetically formed by the oxidative coupling of coniferyl alcohol to the catechol moiety of taxifolin, producing a wide variety of these isomers due to the low stereospecifity of this reaction [4]. All respective flavonolignans occur in the silymarin complex in diastereomeric pairs (always the *trans*-configuration) in proportions of ca. 1:1.

This is why the natural silybin is a nearly equimolar mixture of two diastereomers, silybin A (**1A**), (2*R*,3*R*)-2-[(2*R*,3*R*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one and silybin B (**1B**) (2*R*,3*R*)-2-[(2*S*,3*S*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one (this systematic numbering is not congruous with the customary numbering of the silybin skeleton as given in Fig. 1), whose analytical separation is quite feasible [5], but preparative separation is extremely complicated. The absolute configuration of both silybin A and B was determined few years ago [6,7].

Numerous authors denote both silybin diastereomers "A" and "B" not referring to their configuration—usually according to the order of elution from an HPLC column. This often leads to misunderstandings. Optical rotation ($[\alpha]_D^t$) is the easiest way to assign the absolute configuration of both compounds: natural silybin (a mixture of A and B in the ratio ca. 1:1—usually B is slightly prevalent) has $[\alpha]_D^{23} = +11.4$ ($c = 0.29$, acetone) [8], silybin A has $[\alpha]_D^{23}$ higher than the mixture, +20 ($c = 0.21$, acetone) and silybin B has $[\alpha]_D^{23}$ lower, −1.07 ($c = 0.21$, acetone) [6].

Typical applications, which milk thistle preparations have been used for since ancient times, mostly include treatments of liver diseases and gastrointestinal tract (GIT) problems [9]. Silymarin/silybin and their preparations are currently also advocated for the treatment of cirrhosis, chronic hepatitis, and liver diseases associated with alcohol consumption and environmental toxin exposure [10]. Silybin is considered to be very safe and virtually no serious adverse effects have been reported [11].

Pharmacological, medicinal, and phytochemical literature lists many polemics on the effects of silymarin and/or silybin [11–13]. The main reason for the controversy and uncertainty of their effects is the variable composition of silymarin preparations used in these studies caused by the use of non-standard silymarin (complex extract) and silybin (defined compound) preparations, and confusing these terms [1,14].

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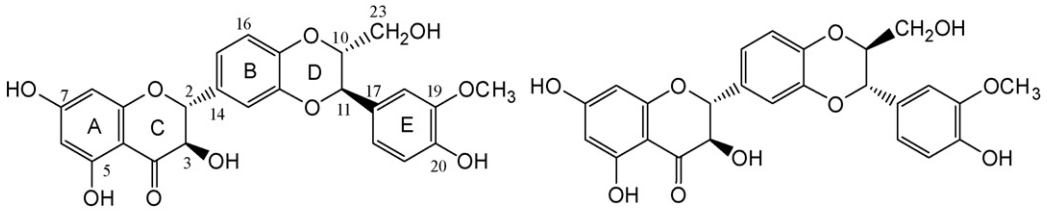


Fig. 1. Diastereomeric silybins (**1A** and **1B**).

Recently, silybin/silymarin has received attention due to its alternative beneficial activities that are not directly related to its hepatoprotective and/or antioxidant (radical scavenging) effects [1,15]. These include mostly anticancer and chemopreventive actions, as well as hypocholesterolemic, cardioprotective, and neuroprotective activities. Moreover, the extent of its application has been broadened to other organ systems besides the liver and GIT, e.g., the treatment of pancreatic problems and balancing glycaemia, treatment of lung problems and kidney diseases, treatment of various disorders of the prostate including adenocarcinoma, and last but not least its utilization in dermatology and cosmetics. This is linked to the discovery of numerous new effects of silybin and its derivatives at the cellular and molecular level, such as, e.g., estrogenic and antiangiogenic activity, modulation of drug transporters (P-glycoprotein), and specific action on DNA-expression *via* the suppression of nuclear factor-κB (NF-κB).

The aspects of optical purity of silybin and other flavonolignans from the silymarin complex have been largely neglected. However, when silybin is used for applications other than as a mere antioxidant in an isotropic milieu (e.g., in solutions reacting with non-chiral radicals), its stereochemistry plays an extremely important role. The studies of respective biological activities on optically pure compounds are also required.

Weyhenmeyer et al. [16] published a study on the pharmacokinetics of silybin diastereomers. However, they used natural silybin (a mixture of silybins A and B) and measured the plasma profiles of – at that time unidentified – diastereomers determined by HPLC. They found that the plasma concentration of one unconjugated silybin diastereomer is three times higher that the concentration of the other one. This difference was presumably caused by a different conjugation rate (glucuronidation) of the respective silybin diastereomers. We later corroborated this assumption by preparing pure silybins A and B glucuronides and by analysis and assignment of the silybin metabolites in humans [17].

The data on the pharmacological activity of pure silybins A and B (*vide infra*) are rather limited. We have recently demonstrated a clear proof that silybin B interacts with the estrogenic receptor, whereas its diastereomer silybin A is inactive [18]. Later, a paper on the activity of pure diastereomers of silybins A and B, isosilybins A and B, and other silymarin components on human prostate carcinoma demonstrated that isosilybin B is the most effective in suppressing the topoisomerase IIα gene promoter in DU145 cells [19]. Silybin B was the most active in the G1 cell cycle accumulation of DU145 cells.

A major problem hampering studies with optically pure silybin is the extremely complicated separation of silybins A and B. So far only separation by HPLC or semipreparatory HPLC has been used to obtain milligram amounts of these compounds. Some authors even claim to have achieved preparatory separation, however they usually end up with some 10–20 mg of pure silybins [20]. Another study [21] focused on the preparatory separation of silybins A and B and other silymarin congeners by HPLC in larger quantities has been recently published. Unfortunately, this is mere repetition and optimization of the previously published HPLC methods on a larger scale (*i.e.* 154 injections/repetitions) and the authors themselves admit that "...it is possible to generate gram quantities of each diastereoisomer within a few months..." [21]. Moreover, this method uses quite expensive separation materials (e.g., HPLC grade RP-18 silica). This is not only unsuitable for the pharmacological production of these compounds but also for preclinical studies in animals.

The proposed procedure consists of the synthesis of 23-*O*-(peracetyl)-β-Gal-silybins, their chromatographic separation, deprotection, and enzymatic hydrolysis yielding the individual silybin isomers (Scheme 1).

2. Experimental

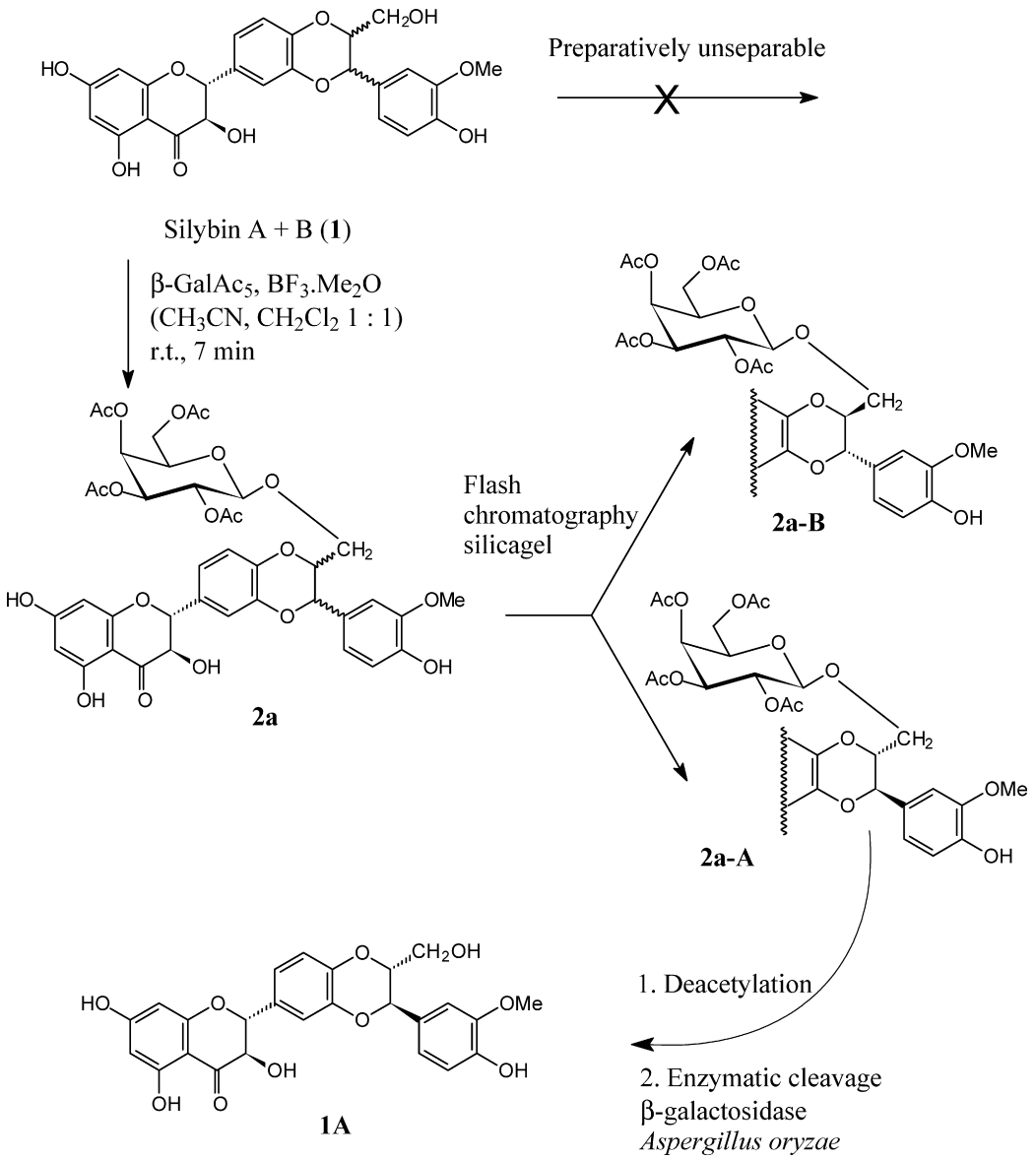
2.1. General methods

^{1}H and ^{13}C NMR spectra were measured in CD_3OD on a Varian VXR-400 spectrometer (399.95 and 100.58 MHz, respectively) for solutions at 25 °C. The residual solvent signal (δ_H 3.33, δ_C 49.3) served as an internal reference. Chemical shifts are given on the δ-scale; digital resolution was 0.0002 and 0.006 ppm, respectively. *J*-values are given in Hz. Carbon signal multiplicity was determined by an Attached Proton Test (APT) experiment. Vendor-supplied pulse sequences were used for 2D NMR (COSY, ROESY, HOM2DJ, HETCOR).

UV and CD spectra were measured on Varian DMS 300 and Jobin Yvon Mark 5 spectrometers, respectively, in the spectral region 200–400 nm for solutions in methanol, 0.5% TFA in methanol, and 0.5% KOH in methanol. The concentrations of the compounds were 25 and 50 mM for UV and CD measurements, respectively.

2.2. HPLC

Monitoring the separation is of paramount importance in all stages. To achieve this goal in quasi-real time, novel fast separation methods were developed for monitoring the purity of silybin and its semisynthetic derivatives. All HPLC analyses were carried out on a Spectra Physics analytical system (San Jose, USA) comprised of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/vis detector. Stationary phase was Chromolith(R) Speed ROD Monolithic column, RP-18e, 50 mm × 4.6 mm, with Chromolith 5 mm × 4.6 mm guard column (all Merck, DE). Mobile phases were: A: methanol–water containing 0.1% TFA (42:58, v/v), B: 100% methanol containing 0.1% TFA, (v/v). A flow rate of 1.2 mL/min was used for the mobile phase at room temperature. The analytes were monitored at 285 nm (scan 220–360 nm), the injection volume was 2–4 μL. All samples were dissolved in mobile phase A to obtain a final concentration of ca. 0.5 mg/mL–typical HPLC separation of silybin A/B and respective glycosides–see Supplementary material (Figs. 1 and 2).



Scheme 1. Chemoenzymatic separation of silybin stereomers *via* its β-galactopyranosides.

For the separation of silybins A and B, isocratic elution with phase A was used (r_t 3.1 and 3.9 min). A gradient elution of 0–10 min 0–40% B starting from phase A was used to separate the isomers of **2a** (r_t 7.4 and 8.0 min). For the typical separation see Figs. 1 and 2 in the Supplementary material.

2.3. Silybin-23-O-β-D-2′,3′,4′,6′-tetraacetylgalactopyranoside (*2a*) (optimized method)

The reaction must be performed under strictly anhydrous conditions, as traces of water substantially lower the yields. Therefore, silybin (kind gift of TAPI Galena, IVAX Pharmaceuticals, Opava, CZ) must be thoroughly dried by azeotropic distillation with toluene to remove crystal-bound water. Dry silybin (**1**) (4.82 g, 10 mmol) and 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (5.85 g, 15 mmol) were dissolved in a mixture of dry CH_2Cl_2/MeCN (200 mL; 1:1, v/v) and $BF_3{\cdot}OMe_2$ (1.83 mL, 20 mmol) was quickly added at room temperature under vigorous stirring under Ar atmosphere. When the reaction was completed (TLC—typically 7 min), the mixture was poured into ice-cold $NaHCO_3$ sat. and twice extracted with 150 mL dichloromethane. The extracts were pooled, dried over Na_2SO_4 and evaporated. The rest of the unreacted silybin and glycosyl donor were removed by flash chromatography on silica gel (toluene/dichloromethane/MeOH 1:10:0.8) to yield crude **2a** (7.4 g, 91%). The reaction has been optimized with respect to the time (see Fig. 3 in Supplementary material) and other parameters to achieve maximum yields.

2.4. Separation of diastereomers *2a-A* and *2a-B*

Final purification and separation of **2a-A** and **2a-B** was achieved by medium-pressure liquid chromatography in a prepacked Lobar column (silica gel, size C, Merck, Darmstad, DE) with the solvent mixture toluene/HCOOH/CH_3COOH (40:0.7:0.7), 9.9 mL min^{-1} with a linear acetone gradient 12–20% (v/v). This separation yielded 1.3 g of **2a-B** (eluting first), 1.4 g of **2a-A** and 3.1 g of a mixture of **2a** A + B (total yield 71%). The mixture A + B can be re-chromatographed to yield another portion of pure silybins A and B. ^{13}C and ^{1}H NMR data of **2a-A** and **2a-B**—see Table 1, Supplementary material.

2.5. Silybin-23-O-β-D-galactopyranoside (**2b-B**)

Acetate **2a-B** (8.12 g, 10 mmol) was dissolved in the mixture Et_3N/MeOH/H_2O 1:8:1 (300 mL, 35 °C, 30 h) and after evaporation, silybin glycosides were again purified by flash chromatography on silica gel (dichloromethane/MeOH/H_2O 8:2:0.5) yielding pure **2b-B** (5.9 g, 92%). Acetate **2a-A** was deacetylated under the same conditions with similar yields.

2.6. Enzymatic cleavage of silybin-23-O-β-D-galactopyranoside (**2b-B**)

Galactoside **2b-B** (0.59 g, 1 mmol) was dissolved in ca. 5 mL of hot MeOH and this solution was diluted with 50 mL of the McIllvain buffer (pH 5), then 20 mg (ca. 160 U) of β-galactosidase from *Aspergillus oryzae* (Sigma) was added and the mixture incubated at 30 °C with shaking. The reaction was monitored using TLC phase IV: EtOAc/MeOH/H_2O/HCOOH 77:13:10:0.5. Silybin B (**1B**) precipitates from the solution as fine yellowish solid. When the precipitation was complete, the mixture was boiled to inactivate the enzyme and volume was reduced to half. The solid was filtered off, washed with water, dried, dissolved in dry acetone and filtered to remove the protein precipitate. The solution was evaporated to yield 401 mg of silybin B (**1B**) as a yellow solid (85%). Its purity can be further improved by recrystallization from MeOH/H_2O. Another portion of silybin (ca. 10% of the additional yield) can be obtained from the filtrate by solid phase extraction using Amberlite XAD-2 non-ionic resin (Sigma) eluted with acetone.

Silybin B (**1B**) d.e. ~99%, $[\alpha]_D^{25}$ = +6.3 (*c* = 0.223, acetone) ^{13}C and ^{1}H NMR data see Tables 1–3 Supplementary material.

Silybin A (**1A**) was obtained by analogous procedure from respective galactoside by enzymatic cleavage as above in the yield of 82%, d.e. ~97%, $[\alpha]_D^{25}$ = +13.7 (*c* = 0.218, acetone) ^{13}C and ^{1}H NMR data see Tables 1–3 Supplementary material; (Scheme 1).

3. Results and discussion

3.1. Synthesis of silybin glycosides

The separation of diastereomeric silybins A and B at preparatory scale is complicated by their very similar (sterical) structure. Generally, the extremely low solubility of silybin in water (ca. 400 mg/L) and in organic solvents makes any chromatographic method difficult. The solubilization of silybin, containing three phenolic groups, in alkaline solutions is not applicable due to its fast base-catalyzed oxidation to 2,3-dehydrosilybin [22].

A suitable derivatization of silybin should (*i*) improve its solubility, (*ii*) increase the "sterical difference" between the A and B diastereomers to facilitate their separation, (*iii*) enable a simple deprotection compatible with silybin antioxidant and antiradical nature and finally (*iv*) be safe in terms of the toxicity of the reagents and solvents. Economical aspects considering future scale-up should not be neglected either.

A series of various silybin C-23 glycosides were prepared for our studies aiming at more soluble and bioavailable silybin derivatives. During purification procedures by flash chromatography on silica gel, we observed a partial separation of the respective acetylated silybin glycosides, which was promising for the preparative separation the diastereomeric pair.

Besides β-galactopyranosides and β-glucopyranosides we attempted also preparation of α-mannopyranosides of silybin using analogous procedure described in methods. The reaction was rather slow (over 24 h) and gave rather complex reaction mixture with low yields of the products. After partial purification we did not observe any traces of separation of respective silybin-α-mannopyranosides, therefore, we concentrated further to galactosides and glucosides, which are prepared – besides better yields – from considerably cheaper sugars.

Therefore, we had to develop a robust synthetic method for the synthesis of silybin β-D-galactopyranoside, and to optimize a preparatory separation method preferably using unmodified silica gel. Finally, a suitable deprotection method had to be developed to yield pure silybins A and B.

First attempts with the Helferich reaction using $Hg(CN)_2$ as a catalyst and α-bromoperacetylgalactose produced reasonable yields of **2a** (up to 75%) after solvent system optimization (nitromethane/toluene 14:11). However, despite extensive purification the final product always contained traces of mercury (polyphenols form complexes with various metal ions), therefore this method was not acceptable. The Koenigs-Knorr reaction using the same glycosyl donor and Ag_2O as a catalyst in dioxan gave a rather low yield (under 10%) of acetylated silybin glycoside. The variation of catalysts: Ag_2CO_3 (Fetizon reagent), $AgClO_4$, $AgNO_3$ and solvents did not improve the yield and in some cases (Fetizon r.) silybin oxidation by Ag^+ was observed. Phase-transfer glycosylation with tetra-*n*-butylammonium hydrogensulfate or benzyltriethylammonium bromide catalysis (α-bromo-penta-*O*-acetylgalactose as glycosyl donor, solvent $CHCl_3$/0.1 M Na-borate buffer, pH 10.8, 52 °C, 4 h) yielded approx. 70% of an acetylated glycoside. However, the fragment ions in the aglycone part of the mass spectrum of the reaction product were of 2 amu lower than those of the parent compound, indicating the silybin oxidation to 2,3-dehydrosilybin under alkaline (pH 10.8) conditions. Finally, glycosylation with Lewis acid catalysis ($BF_3{\cdot}Me_2O$, TMSOTf, $SnCl_4$) was tested using β-D-galactose pentaacetate as a donor. $BF_3{\cdot}Me_2O$ as the catalyst (promoter) gave the best yields (60–80%), whereas the yields were under 20% with the other two catalysts. The reaction with $BF_3{\cdot}Me_2O$ was optimized (glycosyl donor, promoter ratio, solvents, conditions) to achieve a final yield of **2a** of approx. 90% (for the optimized conditions, see Methods). Although it is possible to use both α- and β-galactose peracetates or a mixture of them without influencing their product stereoselectivity (yielding exclusively the β-anomer) the best yields and the lowest side product formation were achieved with pure β-anomer of the peracetylated sugar. This procedure is acceptable from the point of toxicity (no heavy metals), it is fully scalable and the reagents are relatively cheap and easy accessible. Peracetylated silybin glycosides are highly soluble in common organic solvents. Silybin-23-*O*-β-D-glucopyranoside was synthesized by an analogous method (data not shown).

3.2. Preparative separation of optically pure silybin glycosides

Separation experiments were performed with silybin β-D-galactopyranoside (**2a**) due to its slightly better separation parameters compared to its β-D-glucopyranoside analogue.

The crude reaction mixture was first purified to remove unreacted sugar donors and major impurities by flash chromatography on silica gel (system I: toluene/dichloromethane/MeOH 1:10:0.8). Crude peracetylated silybin glycosides A and B were separated by another chromatography system II (toluene/acetone/HCOOH/CH_3COOH 40:10:0.7:0.7), which was extensively optimized, mainly to achieve separation of the respective isomers A and B (**2a-A** and **2a-B**). The silybin B galactoside (**2a-B**) elutes first, followed by the respective A derivative (**2a-A**). The separation is rather critical and minor changes in the elution system (humidity) may even lead to an unexpected interchange of the elution order of diastereomers. Therefore, the identity of isolates has to be always verified, preferably by optical rotation. An alternative mobile phase for separation giving reasonable diastereomer separation is the system III (EtOAc/hexane/HCOOH 60:40:1.5).

Table 1
Enzymatic hydrolysis of silybin-23-*O*-β-D-galactopyranoside (**2b**).

β-Galactosidase (source)	Hydrolysis of **2b**[b]
Penicillium brasilianum CCF 2155[a]	++
Penicillium brasilianum CCF 2171	++
Penicillium chrysogenum CCF 1269	++
Penicillium oxalicum CCF 1959	+++
Aspergillus flavipes CCF 1895	++
Aspergillus flavofurcatis CCF 107	+
Aspergillus flavofurcatis CCF 3061	++
Bacilus circulans (Biolacta, Daiwa Kasei, K.K.)	++
Kluyveromyces lactis (Godo YNL, Godo Shusei Co.)	±
Aspergillus oryzae (Sigma)	+++
Sulfobolus solfataricus	+

[a] CCF strains are deposited at the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic. For the cultivation and production of extracellular enzymes, see our previous papers [23,24].
[b] Readout after 1 h (pH 4.5, citrate-phosphate buffer 0.1 M): ± traces of the product; + 5–10%; ++10–50%; +++ 100% means no traces of remaining **2b**.

The difference in the R_f of both diastereomers (phase II, TLC plates Silica gel 60 F_{254} Merck) is rather small (**2a-B** 0.22, **2a-B** 0.19), therefore the chromatography provides ca. 30% of the pure B derivative **2a-B**, 30–40% of the A + B mixture and ca. 20% of the A derivative **2a-A**. The purity of the B derivative is always higher (first peak) than the second peak (A). Re-chromatography of the mixture **2a-B** + **2a-A** yields another portion of pure diastereomers. Extremely careful performance of the chromatography and thorough fraction analysis is vital for a successful separation.

Among the various silica gel types tested [Silicagel 60 40–63 μm (Merck); Chromagel (SDS, France) and Lobar prepacked silicagel columns (Merck)]; the Lobar columns exhibited the best performance. However, the separation on both Silicagel 60 40–63 μm (Merck) and Chromagel were acceptable, depending strongly on the quality of column packing.

Deacetylation of **2a** to free glycoside (**2b**) can be performed by the classical Zemplén procedure (MeONa/MeOH). However, there is a risk of a base-catalyzed oxidation to 2,3-dehydrosilybin since MeONa has to be added in excess due to the acidity of silybin. To avoid this reaction, the compounds were deacetylated in the mixture Et_3N/MeOH/H_2O 1:8:1 (r.t., overnight). The reaction mixture can then be evaporated to yield pure glycoside **2b** with a virtually quantitative yield.

3.3. Enzymatic cleavage of silybin glycosides

The final step is a deglycosylation to yield pure silybin A or B. Acid cleavage is an option, but the products are always contaminated with impurities and the yields are lower because of decomposition, polymerization, and oxidation. An alternative enzymatic method using β-galactosidase yields pure silybin. We tested a series of various galactosidases to achieve optimal performance (rapid and quantitative reaction). We tested several commercial β-galactosidases: *Bacilus circulans* (Biolacta, Daiwa Kasei, K.K., Hyougo, Japan), *Kluyveromyces lactis* (Godo YNL, Godo Shusei Co., Ltd., Tokyo, Japan), *A. oryzae* (Sigma), then the thermophilic enzyme from *Sulfobolus solfataricus* (kindly provided by Dr. A. Trincone, Napoli, IT), and a series of fungal extracellular β-galactosidases from a library of glycosidases from the Laboratory of Biotransformation (Inst. Microbiol., Prague) (Table 1) [23,24].

4. Conclusions

An optimized high-yield method for silybin glycosylation was developed. Peracetylated silybin glycosides were separated by silica gel low pressure chromatography to obtain optically pure silybins A and B glycosides. The respective glycosides were after deprotection hydrolyzed by the β-galactosidase from *A. oryzae* to yield silybin A and B in gram amounts. This procedure can produce approx. 10 g of silybin A and B within one week. The method does not use toxic or dangerous chemicals and is fully scalable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.07.013.

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