Separation of Flavonoids from *Aleurites moluccana* Leaves Using Chitosan Modified with Heptaldehyde

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Heptaldehyde-modified chitosan (heptyl-chitosan, CH-Hp) was investigated as adsorbent for chromatograhic separation of the flavonoids from *A. moluccana.* The amount of 2″-*O*-rhamnosylswertisin isolated (30.0 mg) was approx. twice as high as swertisin (17.5 mg). The improved surface hydrophobicity effected by the heptyl groups promoted the separation of flavonoids. From the results obtained, CH-Hp seems to be more suitable for separation of glycosylated flavonoids than other flavonoids. Thus, modified chitosan described here can be used for hydrophobic interaction chromatography as sucessfully illustrated with flavonoids.

Key words: Chitosan-heptaldehyde, Chromatography, Swertisin, 2″-*O*-Rhamnosylswertisin

Introduction

Aleurites moluccana (L.) Willd. is an tree which is endemic to India and which has been introduced in Brazil, where it is known by the popular name “nogueira-da-índia” or “nogueira-de-iguape”. This plant belongs to the Euphorbiaceae family, which includes 300 genera and about 7000 species (Gnecco *et al.*, 1996). *A. moluccana* has been extensively used in folk medicine for the treatment of ulcers, headache, fevers, diarrhea and hypocholesterolemia (Duke, 1991). Preliminary evaluations of this plant in our laboratories have demonstraded its antinociceptive and hypolipidaemic effects (Meyre-Silva *et al.*, 1998; Pedrosa *et al.*, 2002). Chemically, we have isolated hydrocarbons, sterols, flavonoids and triterpenes, which have contributed to the analgesic activity of the leaves in previous reports (Meyre-Silva *et al.*, 1997, 1999).

Chitosan, obtained by partial desacetylation of chitin, has attracted considerable attention as a matrix for chromatographic support. Modified chitosan is widely used as stationary phase for normal-phase, reverse phase, chiral phase, hydrophobic interaction and other chromatographic methods (Rodrigues *et al.*, 2000; Saito *et al.*, 2002; Senso *et al.*, 1999; Wang *et al.*, 2002).

Modified chitosan is usually prepared using the technique which involves the reaction between the NH_2 group of polymer and aldehyde, *N*-aryl or *N*-alkyl chloride and aryl isocyanates, to obtain carbamates, esters or alkylamines (Senso *et al.*, 2000).

We have previously reported the use of chitosan modified with benzaldehyde for the separation of flavonoids from *A. moluccana* (Girardi *et al.*, 2003). Extending our studies on this field, this work describes the evaluation of *N*-heptaldehyde modified chitosan as a matrix for the separation of flavonoids from *A. moluccana.*

Material and Methods

Plant material

A. moluccana was collected in Tijucas, in the South of Brazil and a voucher specimen was deposited at the Barbosa Rodrigues Herbarium, Itajaí under number VC Filho 001.

A methanolic extract was obtained after maceration with methanol at room temperature for 10 d (600 g dried leaves). This extract was concentrated and then successively partitioned with hexane, dichloromethane, and ethyl acetate, respectively. The ethyl acetate fraction, rich in flavonoids, was dried at room temperature (1.2 g) and selected for futher studies.

Preparation of the stationary phase

The chitosan derivative was synthesized through Schiff’s reaction using a modified conventional method (Girardi *et al.*, 2003). A methanolic dispersion of chitosan (5.0 g) was continuously stirred and refluxed with heptaldehyde (Aldrich) (18.5 g) for 72 h. The polymers were purified in a Soxhlet apparatus with acetone to remove excess heptaldehyde. In order to hydrogenate the product of the Schiff base heptaldehyde modified chitosan (CH-Hp) was treated with 100 ml (2.0% in water) cyanoborohydride (Aldrich) overnight. The solid was washed with water to remove the excess cyanoborohydride, filtered and dried at 60 °C. The material obtained was characterized by IR, NMR and potentiometric titration (Brugnerotto *et al.*, 2001; Rinaudo *et al.*, 1993). The IR spectra were obtained using KBr disks on an IR-FT Bomem MB-100 spectrophotometer (Quebec, Canada). The polymer was solubilized in CD_3COOD/D_2O (3% w/w) and a ^1H NMR spectrum (200 MHz) was obtained in on a Bomem 200 spectrophotometer. The titration was carried out using an ORION pH-meter, model A920.

N-Acetylation of CH-Hp

In order to avoid the NH_2 interaction with flavonoids, acetic anhydride was used for acetylation of NH_2 free CH-Hp. CH-Hp (5.0 g) was suspended in 100 ml of methanol and 50 ml of acetic anhydride (6.0 g). After stirring at roon temperature for 24 h, the resulting product was isolated by filtration and washed with methanol and water. Finally the CH-Hp was dried at room temperature (Morsch *et al.*, 2002).

Chromatography

150 mg of the ethyl acetate fraction, containing the flavonoids, was chromatographed on a column (2.0 × 30 cm) using 3 g of CH-Hp eluted with a $CHCl_3/MeOH$ gradient and fractions of 5 ml were collected. After monitoring by thin layer chromatography (TLC) eluted with $CHCl_3/MeOH$ (70:30 v/v), the fractions which showed a positive reaction with $FeCl_3$ were combined.

The purity of all the isolated substances was examined by TLC using precoated plates having a 0.25 mm layer of Merck silica gel 60 HF_{254} and eluted with $CHCl_3/MeOH$ (85:15 v/v). The compounds were detected by spraying with $FeCl_3$ (2% in ethanol) solution. The compounds were identified by direct comparison with authentic samples previously isolated from *A. moluccana* (Meyre-Silva *et al.*, 1999).

Results and Discussion

Heptaldehyde was introduced to chitosan by reductive amination. The condensation reaction of a primary amino group with a carbonyl group results in an unstable imino bond by the elimination of a water molecule. In order to hydrogenate the product of the Schiffbase reaction, CH-Hp was treated with cyanoborohydride, resulting in a polymer more stable to the hydrolysis process.

The amount of NH_2 free groups after the reaction with heptaldehyde was 54% which was calculated through titration. Therefore the substitution degree was 0.2, and the *N*-acetylation degree of the CH-Hp, after the acetylation process, was 0.98 (determined by potentiometric titration). The probable structure of the CH-Hp is shown in Fig. 1A.

The infrared spectrum of CH-Hp (not shown) presents two strengthening absorption bands at about 2900 cm^{-1} ($\nu_{C\text{-}H}$ of CH_2 group) and 1400 cm^{-1} ($\delta_{C\text{-}H}$ of CH_2 group) (Brugnerotto *et al.*, 2001; Silverstein *et al.*, 1994).

The ^1H NMR spectrum (not shown) presents peaks at 0.8–1.0 ppm attributed to the proton of

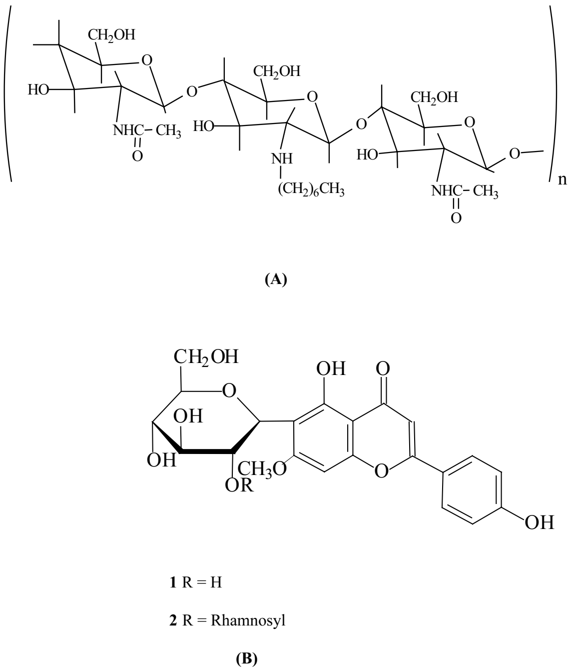

Fig. 1. (A) Chemical structure of CH-Hp. (B) Molecular structures of flavonoids isolated from *A. moluccana*: swertisin (**1**), 2″-*O*-rhamnosylswertisin (**2**).

$-CH_3$ and at 2.1–2.5 ppm attributed to the proton of $-CH_2$ of the heptane group on chitosan. This peak is absent in pure chitosan. The assigment was made by direct comparison with the reported data (Brugnerotto *et al.*, 2001; Silverstein *et al.*, 1994).

Recently, chemically modified chitosan has also been used. Different funtional groups (such as valeraldehyde and 7-ethyloctadecanedioic acid diglycidyl ester) have been chemically introduced into chitosan These modified chitosans have excellent hydrophobicity, and they also cause higher separation than their unmodified analogues (Wang *et al.*, 2002; Saito *et al.*, 2002). Such results have been attributed to an decrease in surface polarity due to the reduction of the amount of free NH_2.

The present work explores the behaviours of CH-Hp as a new adsorbent for chromatographic separation of the flavonoids identified as swertisin (**1**) and 2″-*O*-rhamnosylswertisin (**2**), (Fig. 1B)

Table I gives the yields of the two flavonoids in different adsorbents. The results indicate that the yields are improved with the decrease of free NH_2 groups. Compared with chitin or full *N*-acetylated chitin (Table I) it is clearly demostrated that the dominant contribution to the separation efficiency of these flavonoids is due to the hydrocarbon chain. The retention of flavonoids is the result of a reversed-phase mechanism (nonpolar Van der Waals interactions between phenolic group and adsorbent). Separation for more polar compounds is better if CH-Hp is used, as the hydrocarbon chain makes the surface hydrophobic.

Table I. Efficiency of different support studied in the separation of flavonoids of an ethyl acetate extract *from A. moluccana* (150 mg).

Support	Swertisin [mg][a]	(%)	2″-*O*-Rhamnosylswertisin [mg]	(%)
Chitin[b]	11.2	8.0	10.9	7.2
Chitin-100[b]	12.8	13.5	15.8	10.5
Silica gel[b]	3.0	2.3	9.2	6.1
CH-Hp	17.5	11.6	30.0	20.0

[a] Means of two experiments.

[b] Morsch *et al.*, 2002.

Another interesting result was the high level of compound **2** that was obtained if CH-Hp was used as adsorbent. In chitin and full *N*-acetylated chitin the main chromatographic separation mechanism consists of the formation of a hydrogen bond between the compounds and the acetamide or NH_2-free group. On the other hand **2** is more polar than **1** and the interaction with the adsorbent is larger, resulting in its retention in the chromatography column. When CH-Hp is utilized as adsorbent, the formation of the hydrogen bond appears to be less important compared with the hydrophobic interaction.

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