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Lipase mediated separation of triterpene structural isomers, α- and β-amyrin

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ABSTRACT

Pentacyclic triterpenoids α- and β-amyrin possess a wide range of biological and pharmacological activities. High structural similarity between these two structural isomers makes their chromatographic separation an ineffective and tedious choice. In this study, *Candida rugosa* lipase catalyzed separation protocol for the isolation of individual isomers has been developed. In the presence of vinyl acetate as the acyl donor, *Candida rugosa* lipase carried out acetylation of β-amyrin more efficiently as compared to α-amyrin leading to a kinetic separation. The conditions of transesterification reaction were optimized systematically, which was utilized to separate α- and β-amyrin from a mixture obtained from the latex of *Plumeria obtusa*.

Triterpenoids are known for their diverse biochemical and pharmaceutical properties.[1–6] In specific, α/β-amyrin, structurally characterized as pentacyclic triterpenoids, are formed through cyclization of 2,3-oxidosqualene catalyzed by triterpene cyclase.[7,8] α-, β-amyrin and their derivatives are shown to possess a broad spectrum of biological and pharmacological activities including anti-inflammatory,[9,10] anti-nociceptive,[11] insecticidal,[12,13] anti-depressant,[14] anti-arthritic,[15] gastroprotective,[16] anti-hyperglycemic,[17] anti-microbial,[18,19] and cytotoxic[20] activities. α-/β-Amyrin are structural isomers in relation and possess basic skeletons of two different subgroups of pentacyclic triterpenoids, that is, ursane and oleanane, respectively. The only structural variation in them lies in the E-ring methyl group occupying the position either at C-19 (α-amyrin) or C-20 (β-amyrin) (Fig. 1).

In the last decade α- and β-amyrin and their semi-synthesized derivatives have been investigated extensively for their bioactivities and underlying molecular mechanism.[21–25,14,20,18,15] Unfortunately, many of the studies have been carried out with a mixture of α- and β-amyrin instead of its pure components.[11,14,16,18,20,22,24,25] In fact, the plant resources usually contain a complex mixture of structurally similar pentacyclic triterpenoids with wide variation in their relative abundance. The mixture of pentacyclic triterpenoids shows hardly any chromatographic separation due to their high structural and functional resemblance. As a result chromatographic techniques are truly ineffective and tedious for separating individual components. Very less effort has been exerted till date to purify α- and β-amyrin from their mixture. Literature study has revealed only a HPLC based semi-preparative purification procedure with a long run time.[26]

On the other hand, lipase mediated transesterification is a smart choice for the kinetic resolution of isomeric alcohols. Practically, lipases are preferred biocatalyst for their high stability, broad substrate specificity, independency of coenzymes, and equal efficacy in both the aqueous/organic medium.[27,28] Although, lipases have been widely applied for the kinetic resolution of racemic alcohols or desymmetrization of *meso*-diols and separation of diastereomers or geometrical isomers in a few instances,[29] their application for the separation of structural/constitutional isomers is rare. In this Letter, we have demonstrated the *Candida rugosa* type VII lipase (CAS No. 9001-62-1 and abbreviated as *CRL*) mediated

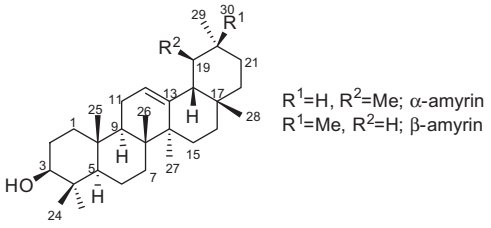

Figure 1. The structures of α- and β-amyrins.

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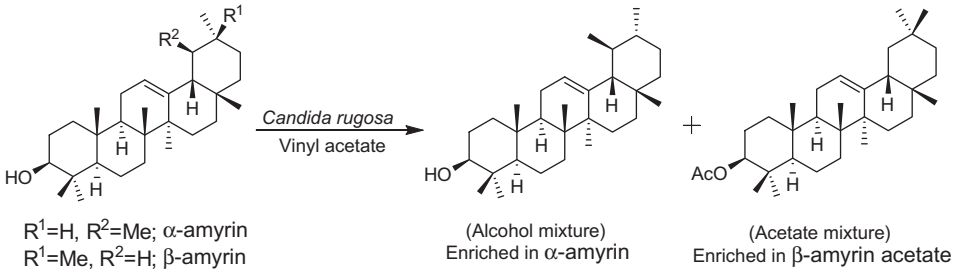

Figure 2. Kinetic separation of α- and β-amyrin mixture by *Candida rugosa* (*CRL*).

transesterification of α- and β-amyrin leading to their kinetic separation. In fact, it is the first Letter on the lipase-catalyzed separation of two structurally isomeric triterpene alcohols (Fig. 2).

Sixteen commercial lipases[30] from various sources were screened for the transesterification of the mixture of α- and β-amyrin using vinyl acetate as the acyl donor in three different solvent systems (*n*-hexane, diisopropyl ether, and toluene). Screening experiments[30] clearly indicated that *CRL* was able to carry out acetylation of β-amyrin in efficient manner compared to its isomer α-amyrin. The GC and GC–MS conditions were standardized for the base line resolution of α- and β-amyrin and their acetates.[31] Other lipases did not show any noticeable transformation on either of α- or β-amyrin.

CRL was able to carry out acetylation of β-amyrin in an efficient manner whereas α-amyrin was found to be very poor substrate for *CRL* mediated transesterification reaction. These observations were further confirmed by the kinetic studies of *CRL* mediated acetylation of pure α- and β-amyrin (Fig. 4). After the incubation period of 48 h, *CRL* converted 38% of β-amyrin (βA-OH) to β-amyrin acetate (βA-OAc) in the presence of vinyl acetate as the acyl donor (Fig. 4A). However, less than 1% of α-amyrin acetate (αA-OAc) formation was observed when α-amyrin (αA-OH) was used as the substrate with similar assay conditions (Fig. 4B). The variable catalytic activity of *CRL* with respect to α- and β-amyrin was efficiently exploited for the kinetic separation of individual isomers from amyrin mixture obtained from *Plumeria obtusa* latex. The mixture of α- and β- (78:22) amyrin (Fig. 3B) was obtained by subjecting the ethyl acetate extract (Fig. 3A) of the *Plumeria obtusa* latex to successive column chromatography.[32]

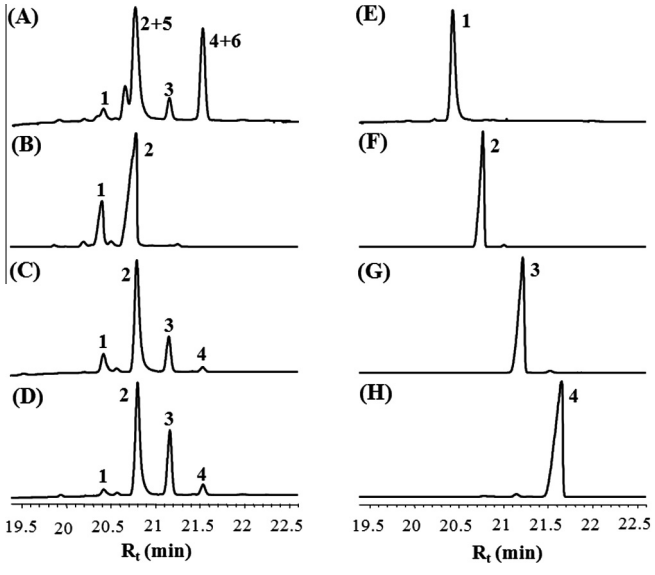

Figure 3. GC-FID chromatograms of (A) *P. obtusa* extract, (B) α- and β-amyrin mixture obtained from *P. obtusa* extract, (C) *CRL* mediated transesterification reaction mixture after 1 day, (D) after 5 days, (E) Pure β-amyrin, (F) Pure α-amyrin, (G) Pure β-amyrin acetate, (H) Pure α-amyrin acetate. Labeling of compounds, **1**: β-amyrin, **2**: α-amyrin, **3**: β-amyrin acetate, **4**: α-amyrin acetate, **5**: Lupeol, **6**: Lupeol acetate.

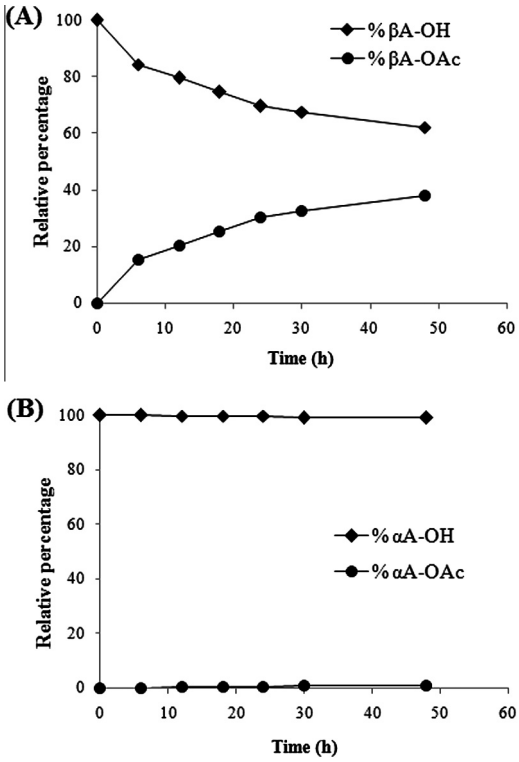

Figure 4. Kinetic studies of *CRL* catalyzed transesterification of pure (A) α-amyrin and (B) β-amyrin. Pure α- or β-amyrin (1.0 mg), *CRL* (12.5 mg), and vinyl acetate (10% of total reaction volume of 2.5 mL) in *n*-hexane were incubated at 37 °C and 200 rpm.

CRL mediated transesterification conditions such as preference of solvent, choice, and effective concentration of acyl donor and concentration of *CRL* were optimized. Among various solvents screened, acyclic ethers (diisopropyl ether, diethyl ether, *tert*-butyl methyl ether) and alkanes (*n*-hexane, *n*-pentane) were found to be better solvent systems for *CRL* mediated transesterification. In fact, *n*-hexane was found to be the most suitable solvent for *CRL* mediated transesterification as indicated by GC analyses. Among eight different acyl donors[33] used for *CRL* mediated transesterification of β-amyrin, only vinyl acetate was used as the acyl donor by lipase system. To determine the optimized concentration of acyl donor, transesterification reaction was carried out with variable proportions of vinyl acetate in *n*-hexane. The percentage of acetylated product (% conversion) was in the same range at various

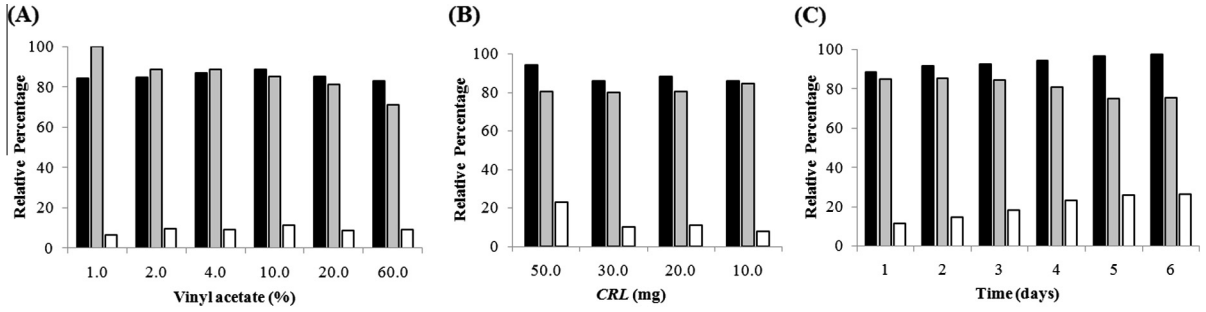

Figure 5. (A) Optimization study of effective vinyl acetate concentration. Amyrin mixture (4.0 mg), *CRL* (50.0 mg), and vinyl acetate (1.0, 2.0, 4.0, 10.0, 20.0, and 60.0% of total reaction volume of 5.0 mL) in *n*-hexane were incubated for 24 h. (B) Optimization study of effective *CRL* concentration. Amyrin mixture (4.0 mg), *CRL* (50.0, 30.0, 20.0, and 10.0 mg), and vinyl acetate (10% of total reaction volume of 5.0 mL) in *n*-hexane were incubated for 96 h. (C) Time course experiment of *CRL* catalyzed separation of amyrin mixture from *Plumeria obtusa*. Amyrin mixture (4.0 mg), *CRL* (50.0 mg), and vinyl acetate (10% of total reaction volume of 5.0 mL) in *n*-hexane were incubated together and monitored at an interval of 24 h. All the reaction mixtures were incubated at 37 °C and 200 rpm throughout. [■ % of α-amyrin in alcohol mixture, ▩ % of β-amyrin acetate in acetate mixture, □ % of total acetylated products in the reaction mixture (% conversion)].

concentrations (1.0%, 2.0%, 4.0%, 10.0%, 20.0% and 60.0% total reaction volume, respectively) of vinyl acetate during 24 h of incubation period although at 10.0% concentration of vinyl acetate, slightly higher level of acetylated product formation was observed (Fig. 5A). Interestingly, lower vinyl acetate concentrations led to the higher abundance of βA-OAc in the acetate mixture and this condition can be utilized for the purification of β-isomer.

The effective concentration of *CRL* was optimized by monitoring the reaction mixture containing 10.0, 20.0, 30.0, and 50.0 mg of *CRL*, respectively, along with 4.0 mg of amyrin mixture as substrate and 10% of vinyl acetate as the acyl donor. The progress of the reaction was monitored by GC and GC–MS analyses[31] of the aliquots drawn at the interval of every 24 h. Although after 24 h all the sets showed similar progress and enrichment, at the end of 96 h highest percentage of conversion (23.0%) was achieved with 50 mg/4.0 mg amyrin mixture. The highest abundance of α-amyrin in the alcohol mixture (94.4%) was attained with same *CRL* concentration (Fig. 5B). Time course experiment was performed to monitor time dependent progress and enrichment of *CRL* mediated transesterification product (Figs. 3C, D and 5C). After 6 days of incubation period, percentage conversion reached highest (26.3%) with 97.5% purity of α-amyrin in alcohol mixture. Prolonging the incubation period did not yield noticeable changes in the levels of α-amyrin (Fig. 5C).

In conclusion, *Candida rugosa* lipase (*CRL*) catalyzed separation protocol was developed for the separation of structurally isomeric triterpene mixture of α- and β- amyrin.[34] *CRL* was able to carry out acetylation of β-amyrin into β-amyrin acetate in a selective and efficient manner at lower concentration of vinyl acetate in the early stage of incubation period (24 h). On the other hand, α-amyrin was obtained by incubating *CRL* with α- and β-amyrin mixture for prolonged incubation period (6 days). *n*-Hexane and vinyl acetate were found to be better organic solvent and acyl donor, respectively, for the *CRL* mediated transesterification. This separation procedure might be useful for the large-scale separation of α- and β-amyrin for their detailed biological and commercial applications.

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30. *Lipases screened:* CAL-B (Novozyme 435), CAL-A, Amano lipase M (from *M. javanicus*), Amano lipase A (from *A. niger*), Amano lipase PS (immobilized on diatomite), Amano lipase G (from *P. camemberti*), Amano lipase AK (from *P. fluorescens*), Lipase from *T. lanuginosa*, Lipase from *P. camemberti*, Lipase from wheat germ, Lipase from *C. rugosa*, Lipase from Porcine pancreas type-II, Lipase from *C. rugosa* immobilized on immobead 150, Lipase from *R. niveus*, Lipase from *R. arrhizus*, Lipase from *C. rugosa* type-VII. *Screening condition:* Amyrin mixture (1.0 mg), lipase (5.0 mg), and vinyl acetate (10% of total reaction volume of 2.0 mL) in various solvents were incubated at 200 rpm for 24 h at a temperature directed by manufacturer's instructions. In time course studies and screening experiments, the aliquot of reaction mixture (100 μL) was drawn, filtered, and diluted to 0.5 mL (total volume) using *n*-Hexane. 1.0 μL of this diluted reaction mixture was subjected to GC/GC–MS analyses.

31. *GC and GC-MS conditions:* GC-Instrument: Agilent 7890 attached with hydrogen flame ionization detector, column: HP-5 (30 m × 0.32 mm × 0.25 μm, J & W Scientific), carrier gas: Nitrogen with flow rate 1.0 mL/min, temperature program: Initially the column was maintained at 150 °C for 2 min followed by a temperature gradient from 150 °C to 320 °C at 10 °C/min and finally the temperature was maintained at 320 °C for 11 min, injector: 300 °C, detector: 280 °C, split ratio: 1:10. Retention time (R_t in min): 20.4 (β-amyrin), 20.8 (α-amyrin), 21.2 (β-amyrin acetate) and 21.6 (α-amyrin acetate). GC–MS instrument: Agilent 7890A GC coupled with 5975C mass detector, column HP-5-MS (30 m × 0.25 mm × 0.25 μm, J & W Scientific), other conditions: similar as GC.
32. *Extraction of α- and β-amyrin mixture:* Latex (175 g) collected from *P. obtusa* was taken in water (400 mL) and extracted with ethyl acetate (600 mL × 6). Ethyl acetate layers were pooled together and concentrated to get 33 g white solid, which upon fractionation by column chromatography using gradient mixture of ethyl acetate–hexane furnished two fractions. Less polar fraction (8.2 g) contained the mixture of α-, β-amyrin acetate and lupeol acetate whereas more polar fraction (12.3 g) was consisted of deacetylated analogues of same pentacyclic triterpenoids. From the alcohol fraction, mixture of α- and β-amyrin (4.8 g, α:β = 78:22) was obtained through argentation column chromatography.
33. *Acyl donors screened:* Vinyl acetate, Vinyl stearate, Vinyl benzoate, Vinyl laurate, Vinyl propionate, Vinyl cinnamate, Vinyl decanoate and Vinyl butyrate. *Screening condition:* Amyrin mixture (1.0 mg), *CRL* (5.0 mg) and acyl donor (10% of total reaction volume of 2.0 mL) in *n*-hexane were incubated at 37 °C and 200 rpm for 24 h.
34. *Optimized separation protocol: CRL* (200.0 mg) was added to the assay mixture containing α- and β-amyrin mixture (16.0 mg, 78:22, respectively) and 0.5 mL vinyl acetate in *n*-hexane. The total volume of assay mixture was 5.0 mL. This reaction mixture was incubated at 37 °C on a metabolic shaker with 200 rpm for six days. After this incubation period, the reaction mixture was subjected to flash column chromatography on silica gel (60–120 mesh) to obtain α-amyrin (11.0 mg, 97.5%). The acetate fraction contained both α- and β-amyrin acetates. To obtain pure β-isomer, the assay was carried out under the same conditions except the vinyl acetate was 0.05 mL and incubation period was 24 h. At the end of this incubation period, the β-amyrin acetate (1.0 mg, 99.9%) obtained in pure form after subjecting to column chromatography under similar conditions. For large scale purification, the volume of the assay mixture was increased proportionately.