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SHORT COMMUNICATION

Biocatalytic conversion of aloeresin A to aloesin

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Abstract Leaf exudates from *Aloe* species, such as the Southern African *Aloe ferox*, are used in traditional medicines for both humans and livestock. This includes aloesin, a skin bleaching product that inhibits the synthesis of melanin. Aloesin, (a C-glycoside-5-methylchromone) can be released from aloeresin A, an ester of aloesin, through hydrolysis. The objective of the current study was to identify an enzymatic hydrolysis method for converting aloeresin A to aloesin, resulting in increased concentrations of aloesin in the aloe bitters extract. More than 70 commercially available hydrolytic enzymes were screened for the conversion of aloeresin A. An esterase (ESL001-02) from Diversa, a lipase (Novozym 388) and a protease (*Aspergillus oryzae*) preparation were identified during screening as being capable of providing conversion of pure aloeresin A, with the protease giving the best conversion (~100%). It was found that a contaminating enzyme in Novo 388 was responsible for the conversion of aloeresin A to aloesin. This contaminating enzyme, possibly a protease, was able to give almost complete conversion using crude aloe bitters extract, doubling the concentration of aloesin in aloe bitters extract via the hydrolysis of aloeresin A.

Keywords Biocatalysis · *Aloe ferox* · Aloeresin A to aloesin

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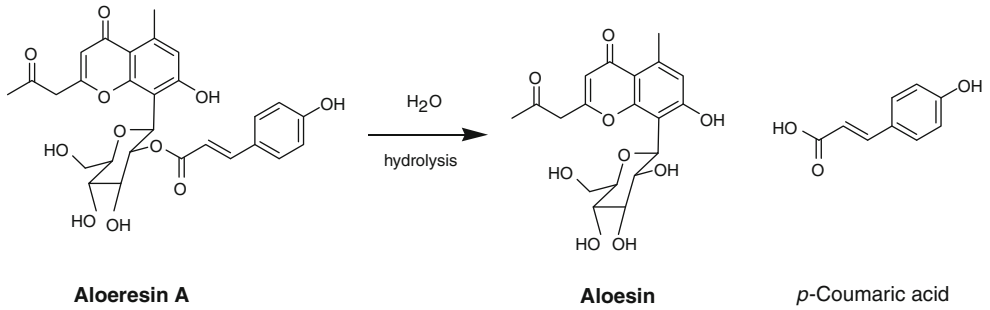
Introduction

Over 300 species of *Aloe* are known, most of which are indigenous to Africa. Leaf exudates from *Aloe* species are used to a great extent in traditional medicines, both for humans and livestock [13, 15]. The main source of African drug aloes is *A. ferox*, a species restricted to Southern Africa. *A. ferox* is a single-stemmed plant that typically grows to a height of approximately two metres with broad fleshy leaves that are dull green to greyish green in colour [12]. Other species of aloe are also used for pharmaceutical, therapeutic, dermatological or cosmetic purposes, particularly *Aloe vera*, which is used extensively in the USA in skin care products, shampoos and health drinks. *A. ferox*, however, produces approximately 20 times more bitter sap, weight for weight [3, 7]. Since *A. ferox* leaves are much thicker and wider, the total yield of bitter sap per leaf is even greater. The bitter sap is collected and excess water is removed through boiling to yield the bitter crystals [1]. The chemical composition of the bitters consists of anthrones and chromones. The diastereomers aloin and barbaloin (also known as aloin A and B) form part of the anthrones, while aloesin and aloeresin A are chromones. Anthraquinones, naphthalene, alkaloids and other minor compounds are also present [13, 14]. *Aloe ferox* bitters sap is a valuable source of aloesin (2-acetonyl-8-glucopyranosyl-7-hydroxy-5-methylchromone), a clinically proven skin lightener and



Scheme 1 Conversion of aloeresin A to aloesin

UV-B protectant [6, 16], and aloin ((10S)-10-glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10H)-anthracenone), an FDA-approved natural laxative. Many methods exist for the isolation of particular components from the aloe [5, 8].

Pharmaceutically, aloesin bleaches skin by reducing melanin formation through competitive inhibition of tyrosinase at noncytotoxic concentrations [2, 5]. Skin bleaching is important in the cosmetic treatment of hyperpigmentation, which is the result of exposure to ultraviolet light, certain drugs and chemicals, or disease, especially in middle-aged and elderly people [9]. While some agents used as tyrosinase inhibitors are non-natural, irritants, and are slow acting, aloesin is a natural compound that is effective as a pigmentation-altering agent for cosmetic and therapeutic applications [5, 17].

In *A. ferox*, the combination of aloeresin A, aloesin and both epimers A and B of aloin contributes between 70 and 97% of the total dry weight of leaf exudate, in a ratio of approximately 4:3:2, respectively [13]. Since aloeresin A is an aloesinyl ester of *p*-coumaric acid, it should be transformable to aloesin by means of hydrolysis of the ester bond. The yield of aloesin available for isolation can therefore be significantly increased by the hydrolysis of aloeresin A, which is present in large quantities in the plant, to aloesin and coumaric acid (Scheme 1).

However, it was found that this was difficult to accomplish using traditional chemical methods of alkaline hydrolysis, since the ester is not very labile owing to the delocalisation of electrons into the aromatic ring. The presence of acid- and base-sensitive groups on the aloesin and aloin components of the aloe bitters also makes the use of base hydrolysis undesirable due to the formation of unwanted by-products and the consequent reduction in yield. Acid hydrolysis using 5N HCl or H_2SO_4 at elevated temperatures also results in yield losses [11].

Enzyme-based biocatalysis typically operates under milder conditions, and hence may overcome these problems. This study was therefore undertaken to screen and optimise hydrolytic enzymes for possible candidates that could result in the hydrolysis of aloeresin A.

Materials and methods

Aloeresin A was obtained from Univera Inc. (Lacey, WA, USA), and was also purified in our laboratories from *A. ferox* leaf extract from plants grown in the Eastern Cape Province, South Africa. *p*-Coumaric acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). To find a suitable enzyme which could convert aloeresin A to aloesin, different hydrolytic enzymes were tested using commercial enzymes available in kits such as ESL from Diversa (now Verenium, San Diego, CA, USA), Fluka (Milwaukee, WI, USA), and Altus Biologics (Waltham, MA, USA). Other enzymes were purchased or donated as gifts from companies such as Novozymes SA (Johannesburg, South Africa), Amano Enzyme Inc. (Suqian, China), Nagase & Co. (New York, USA), and Quest International (Carrigaline, Ireland). The hydrolytic enzymes included lipases, esterases, proteases and even an acylase in the Altus Biologics kit. The enzymes eventually optimised were Novozym 388 enzyme, which was a kind gift from Novozymes, ESL 001-02, which was obtained from Diversa (activity 302 units/mL, 1.42 mg/mL total protein and estimated 75% purity), Protease M, which was from Amano, and Bioprotease P conc from Quest International. The ODS2 Spherisorb column (25 cm × 4.6 mm, 5 µm C18) was obtained from Waters (Milford, MA, USA). All other reagents were analytical grade.

Analytical methods

Semiquantitative TLC method

A semiquantitative TLC method was developed as an analytical tool for screening to monitor the relative amounts of aloeresin A, aloesin and coumaric acid in the reaction mixture, using a Camag automatic sampler II, densitometer III and twin-trough development chamber (20 cm) (Camag, Wilmington, NC, USA). The stationary phase that was used consisted of Merck/Sigma RP-18 plates with fluorescent indicator (UV 254 nm) of dimensions 20 × 10 cm by

150 μm thick. The mobile phase consisted of acetonitrile:water [10: 90% (v/v)], and components were separated isocratically. The water was adjusted to pH 2.2 with concentrated phosphoric acid before the addition of the acetonitrile. Densitometry of the plate was conducted in the absorption and reflection mode at a short ultraviolet wavelength of 275 nm. The integration of selected peaks was conducted by means of peak area. The calibration of the standard tracks was performed by linear regression.

HPLC quantitative method

Aloesin, *p*-coumaric acid and aloeresin A were determined quantitatively by HPLC using an ODS 2 Spherisorb column, with gradient elution and UV detection at 297 nm and 30°C. Elution on the column was achieved by initially eluting isocratically with 66% A and 34% B, where eluent A was 0.1% phosphoric acid in water and eluent B was methanol. The gradient was started after 5 min and changed to 24% A and 76% B in 10 min. Aloesin eluted at 3.9 min, *p*-coumaric acid at 9.6 min, and aloeresin A at 10.75 min.

Additional compound analysis was performed using evaporative light scattering detection and mass spectroscopy (ELSD/MS) (Alltech ELSD 2000ES, Waters) [4].

Purification of aloeresin A

Aloe bitters extract (15 mL) was added to 30 mL 10% (v/v) aqueous methanol, and the undissolved material was removed by centrifugation. The methanol–water layer was washed and extracted with 4 × 30 mL ethyl acetate. The ethyl acetate layer was separated and the organic solvent removed under reduced pressure. The resulting precipitate was dissolved in a minimum amount of 2-butanol with heating, slowly cooled to 4°C and left overnight. The precipitate, containing 76% aloin, was removed by filtration with Whatman No 1 filter paper. The organic layer was then washed again to remove residual aloesin. The 2-butanol was subsequently removed under vacuum and 20% aqueous ethanol added to give a final concentration of 30% dissolved solids. The precipitate formed after standing at 4°C overnight was removed by filtration with Whatman No 1 and dried. Analysis on HPLC gave 70% aloeresin A and 11% aloin based on % w/w of total dissolved solids.

Screening of enzymes on commercial aloeresin A

The ability of commercial hydrolytic enzymes to convert aloeresin A to aloesin and *p*-coumaric acid was tested using 74 commercially available enzymes (lipases, esterases, proteases and an acylase). Purified aloeresin A powder (91% pure from Univera) was used for the screening experiments at a concentration of 10 mg/mL. The substrate was made up by dissolving 1 g aloeresin A in 100 mL 0.075 M Tris pH 8 containing 1 mL Tween 80. The substrate solution (1 mL) was added to each of the 74 commercial hydrolytic enzymes in 2 mL Eppendorf tubes. Approximately 1 mg of powdered enzymes or 20 μL of liquid enzymes were tested. The reactions were maintained at 37°C for 20 h and under agitation at 150 rpm. The samples were then analysed for aloesin and *p*-coumaric acid formation by TLC. In a subsequent screen, the buffer used was a 0.01 M sodium phosphate buffer at the lower pH of 5.5 to eliminate the possibility of alkaline hydrolysis.

Preliminary optimisation of biocatalytic aloesin A conversion

Reactions were performed in triplicate for the enzymatic hydrolysis of aloeresin A to aloesin in aloe bitters extract at the 3 mL scale. Enzyme concentration was varied between 10 μL and 1 mL, aloe bitters extract between 50 μL and 200 μL, Tween 80 concentrations of 0, 1 and 10% v/v were used, and the reaction pH was set to 6, 8, or 10 by using Tris, sodium phosphate, or sodium carbonate/bicarbonate, respectively (0.001–1 M). Reactions were maintained at 37°C with stirring at 250 rpm for 22–24 h. In order to stop the reaction, the reaction or sample volume of 3 mL was diluted to 25 mL with water:methanol:THF (20:40:40), and the samples were analysed on HPLC.

ESL 001-02 esterase was run at 70°C and also reacted in the presence of additional buffers: MOPS, glycylglycine, HEPES, and triethanolamine.

Aspergillus oryzae protease (available commercially as Protease M from Amano or Bioprotease P conc from Quest International) was added as a solid (1–50 mg). Buffers used were sodium phosphate, borate or carbonate at pH 4–7. Acetone 0–50% v/v was also tested as a co-solvent. Following the initial experiments, the reaction conditions used were 2–20 mg of enzyme in 0.01 M sodium phosphate at pH 5.5 and 37°C, with agitation at the 6 mL scale.

In the case of Novozym 388, Tween 80 addition at 50–100 μL was also investigated.

Statistically designed experiments with Novozym 388

A two factorial design on Design-Ease (Stat-Ease Inc., Minneapolis, MN, USA) was created to test seven factors that may influence the aloeresin A to aloesin conversion. A ¼ fractional design was created with 32 experiments and two midpoints. The factors tested were: (1) temperature at 25°C and 37°C, (2) pH at 7 and 9, (3) Tween 80 at 0 and 5%, (4) buffer type using either Tris or sodium phosphate, (5) buffer concentration at 0.1 M and 1 M, (6) substrate concentration at 100 μL aloe bitters extract and 500 μL aloe bitters extract, and (7) enzyme concentration at 100 μL and

400 μL. Reactions were done at the 3 ml scale and run for 22 h with agitation in the multireactor at 1600 rpm. The reactions with the highest yields were confirmed at the larger scale in a 50 mL jacketed reactor.

Application of immobilised Novozym 388

Commercial immobilised Novozym 388

Novozyme provides Novozym 388 in an immobilised form on a phenolic-type anion exchange resin which is commercially available as Lipozyme RM IM. Reactions were performed using the immobilised enzyme to compare it to the free enzyme performance. Different substrate-to-enzyme ratios, as well as temperatures ranging from 37 to 70°C, were tested. Larger-scale reactions in which the pH was controlled at 8 and 7.3 were also done.

Sodium alginate immobilisation of Novozym 388

Novozym 388 was entrapped in sodium alginate using a published method [10] that was slightly modified. Sodium alginate (10 g) was agitated with 250 mL of water at 50°C for 1 h on a shaker at 200 rpm. To approximately 50 mL of this solution, 10 mL liquid Novozym 388 (batch 0020) were added and agitated at 40°C for another hour. The suspension was transferred to a 500 mL separating funnel with an outlet 6 mm in diameter. The suspension was added dropwise to a stirred solution of 10% aqueous calcium chloride (500 mL) in a 2 L flask. The addition took approximately an hour. The beads were washed with water and then a portion of the immobilised enzyme (7 mL, which represents 1 mL liquid enzyme preparation) was incubated with 100 μL aloe bitters extract in 5 mL 0.1 M Tris buffer pH 8. The reaction was agitated on a shaker at 150 rpm and 25°C for 70 h. To test the recycling of the enzyme, the immobilised enzyme was washed three times with deionised MilliQ water and then once with the Tris buffer pH 8, before adding fresh buffer and substrate.

Postreaction purification of aloesin from aloe bitters extract

Three column purifications on Silica 60 gel 0.2–0.5 mm were performed sequentially. The first two columns were packed in ethyl acetate, and the first one eluted with decreasing concentrations of ethyl acetate and increasing concentrations of methanol. The eluent containing aloesin was transferred to a second column and eluted stepwise with ethyl acetate and acetone in different ratios. A third column was run using pure acetone to elute the aloesin in a pure form. The aloesin was then recrystallised from ethanol and diethyl ether (first time) and pure ethanol (second time), with diethyl ether washes.

Results and discussion

Purification of aloeresin A

The purification of aloeresin A was problematic, as some colour compounds co-migrated with the aloeresin A when analysed qualitatively on TLC. Analysis by ELSD/MS revealed that the aloeresin A was fairly pure (70% w/w) and that a compound with a sugar component (preliminary identified as galactose) may have been responsible for the colour component resulting from polymer formation during heating of the bitter sap. The original aloe sap and aloe bitters were also analysed to determine if any new compounds, especially polymers, are formed during the process, as the reaction mixtures always rapidly turned dark brown to black. No new peaks were detected. It is therefore possible that the sugars in the sap or bitters may be responsible for the dark colour, due to possible polymer or tar formation during heating.

Screening of the commercial enzymes

In the first screening experiment, four enzymes were found that were able to hydrolyse aloeresin A to aloesin at pH 8 with various degrees of completion. The *Mucor miehei* lipase yielded a small amount of product, while *A. oryzae* protease yielded aloesin and *p*-coumaric acid, plus an additional peak at pH 8. This unwanted by-product could be eliminated by performing the reaction at pH 5.5, as determined in a second screening process. The enzymes ESL001-02 from Diversa and Novozym 388 from Novozyme gave the required products in reasonable amounts at pH 8. A separate screening experiment was done at pH 5.5 to eliminate the possibility of alkaline hydrolysis of the aloeresin A. At pH 5.5, Bioprotease P (conc) from Quest International and Amano Protease M (both *A. oryzae*-based preparations) gave very promising results.

Preliminary optimisation using ESL001-02

The initial experiments determined that using a buffer concentration of 1 M resulted in >60% mole balance loss. Further investigation uncovered that, during heating at 70°C, the buffer caused by-product formation proportional to ionic strength. At 0.001 to 0.01 M buffer, the mole balance problem could be controlled, but no conversion of aloeresin A to aloesin was observed. Higher buffer strength resulted in conversion but caused mole balance problems. Experimental work using ESL001-02 was therefore discontinued.

Hydrolysis with *A. oryzae* protease

An enzyme found during the screening at pH 5.5, the *A. oryzae* protease (Protease M and Bioprotease P), was able to fully convert aloeresin A to aloesin at pH 5.5 using purified aloeresin A. At this buffer pH, molarity and reaction temperature, the reaction mole balance was near 100%, indicating that the reaction conditions did not cause aromatic by-product formation.

The optimisation of conversion using Protease M was attempted on aloe bitters extract as well as pure aloeresin A (91% purity). The best conditions were 4 mg substrate in 37°C 0.01 M phosphate buffer pH 5.5, using 10 mg Amano Protease M per 3 mL reaction mix, wherein aloeresin A was fully converted to aloesin and *p*-coumaric acid after 4 h. The mole balance was close to 99% when using pure aloeresin A as substrate. The optimal conditions for Protease M were the same as for Bioprotease P, but the conversion was complete in 20 h.

However, the conversion of aloeresin A to aloesin in the aloe bitters extract was unsuccessful, and none of the reactions went to completion. The best conversion was a 50% increase in aloesin obtained within 4 h, and no significant increase in conversion was seen after 24 h. Stats-designed experiments were performed in an attempt to enhance conversion, which included testing pH, (pH 4–7), buffer type (borate and sodium phosphate), buffer strength from 0.01 to 1 M, as well as two temperatures: 37 and 50°C. Co-solvent addition was evaluated to aid in the solubility of the substrate. Ethanol, acetone, ethyl acetate, 1-butanol and hexane were all tested. Unfortunately, no improvement in the results was obtained, and high pH values caused severe mole balance losses. Hence, these enzymes were not suitable for crude bitters extract, and an alternative biocatalyst was required.

Optimisation using Novozym 388

The optimised conditions for the reaction was found to be 50 µL aloe bitters extract in 1.95 mL 0.1 M Tris buffer pH 8 and 1 mL enzyme (5 mg as determined by the Biorad assay). In 20 h there was an 80% w/w increase in the aloesin content and a 93% decrease in aloeresin A. The conversion was therefore almost complete, with a mole balance of 95%. Doubling the aloe bitters extract per 3 mL reaction (to 100 µl) and 1 or 2 mL enzyme resulted in a 54% w/w increase in aloesin, but approximately 50% of the aloeresin A was still present. The mole balance for the reaction was 96%. Adding different concentrations of Tween 80 had no influence on the conversion or the mole balance. No conversion was achieved at a lower buffer strength (0.01 M).

Immobilised Novozym 388

In order to recover the enzyme and improve on the techno-economics of the reaction through enzyme recycling, it would be ideal to immobilise the enzyme. The Novozym 388 was therefore obtained in the immobilised form from Novozyme, and it was also immobilised in-house.

Neither of the immobilised enzyme preparations (the commercial Lipozyme RM IM or Novozym 388 entrapped in alginic acid in our laboratories) gave satisfactory conversion of aloeresin A to aloesin in the aloe bitters extract. Both of the immobilised enzymes resulted in large mole balance losses of both the aloeresin A and aloesin, with between 50 and 90% losses depending on the reaction conditions. On further investigation, this turned out to be due to the immobilisation support.

Scaling up the Novozym 388 free enzyme reaction

The reaction was scaled up to 20 mL in a 50 mL jacketed reactor containing 0.33 g aloe bitters extract and run for 48 h at 37°C. During the reaction, aloeresin A decreased from an initial 58 mg to 6 mg, thereby releasing 8 mg of *p*-coumaric acid, while aloesin increased from 36 mg to 78 mg (Fig. 1). The mole balance was calculated to be 95% m/m.

However, the results obtained using other batches of Novozym 388 on pure aloeresin A gave very different and usually poorer results. It was found by comparing gel electrophograms of the enzyme batches that the conversion was linked to the presence of a contaminating enzyme—possibly a protease—in the Novozym 388 preparation, and not the main lipase. There were approximately 5 mg of protein per mL enzyme preparation and four distinct proteins could

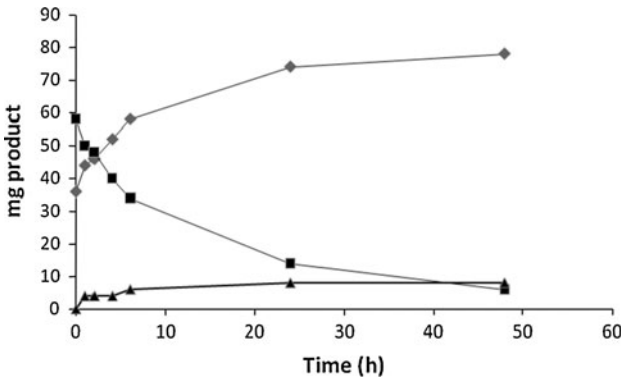
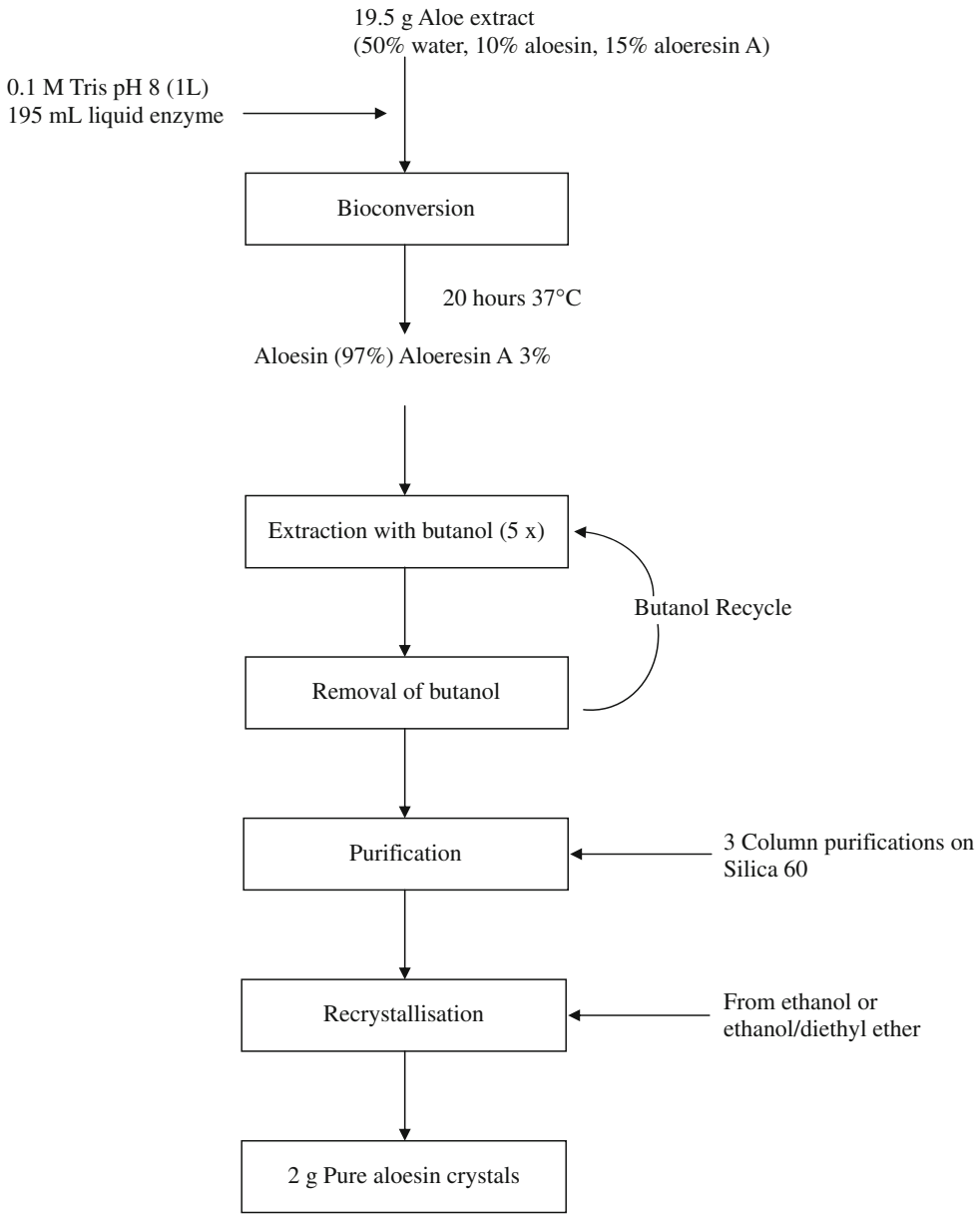


Fig. 1 Time profile for the conversion of aloeresin A to aloesin in an aloe bitters extract, where *filled diamonds* represent the amount of aloesin (mg), *filled squares* represent the amount of aloeresin A (in mg) and *filled triangles* indicate the amount of *p*-coumaric acid in the reaction (in mg). The initial mass of aloesin in the aloe bitters extract was 36 mg prior to enzymatic hydrolysis of aloeresin A



Scheme 2 Aloeresin A conversion to aloesin using the Novozym 388 liquid enzyme

be detected. Each fraction was tested and only one resulted in the conversion of aloeresin A to aloesin.

Postreaction purification of aloesin

The reaction was scaled up in order to obtain a sample of pure aloesin, and the final process is given in Scheme 2. The aloe compounds were extracted five times from the broth using 1-butanol. The extracts were then concentrated and purified on silica 60 gel by means of flash chromatography. The concentrate was passed through three different columns, with the first two removing most of the black contaminants. In the third column, the aloesin was separated from the aloeresin A and then recrystallised. From the initial 19.5 g aloe bitters extract suspended in 1 L 0.1 M Tris buffer pH 8, approximately 4 g of product were obtained following the column fractionations. Following recrystallisation, 2 g of aloesin with a purity of 99% were collected.

Conclusions

Aloesin is a natural skin-whitening compound that is available from aloe plants in small quantities. Aloeresin A (an aloesin ester) is also present in the aloe plant in much higher quantities, and therefore the hydrolysis of aloeresin

A could potentially lead to increased concentrations of aloesin for isolation.

Through a screening process, four hydrolytic enzymes were identified which were able to convert aloeresin A to aloesin and *p*-coumaric acid. The thermostable ESL00-02 from Diversa was initially optimised, but research was discontinued due to high yield losses under the optimum reaction conditions.

Bioprotease P (*A. oryzae*) gave complete conversion in 20 h using purified aloeresin A. *Aspergillus oryzae* protease from Amano (Protease M) provided complete conversion within 4 h with a mole balance closure of close to 100% when using the purified aloeresin A as substrate. However, the enzyme was less effective when applied to crude aloe bitters extract. Hence, a process based on this enzyme would require pre-purification of the aloeresin A to permit complete conversion.

A process using Novozym 388 was developed, but following variable results from different batches of the Novozym 388, it was discovered that the activity of a contaminating enzyme—possibly a protease—in the preparation, and not the main enzyme, was responsible for the conversion. If the contaminating enzyme can be identified and produced, the conversion using this enzyme will be applicable to the conversion of aloeresin A to aloesin from crude aloe bitters extracts, as this enzyme could be applied to aloe bitters extract with more than 90% conversion to the product.

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