Enzymatic Extraction of Pilocarpine from *Pilocarpus jaborandii*

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Received : March 25, 2013 / Revised : April 24, 2013 / Accepted : April 25, 2013

**Introduction**

Many of the alkaloids used as drugs today are from natural sources. These drugs have been modified to produce analogs for clinical use. One of these alkaloids, pilocarpine, is an imidazole alkaloid found in plants of the genus *Pilocarpus* (Fig. 1). Plants of this genus are designated by the name *jaborandi* but only *Pilocarpus microphyllus*, which accumulates the highest pilocarpine content, is considered as true *jaborandi* [17, 22]. *Jaborandi* growing as a shrub is found in the understory of the pre Amazonian rain forest and occurs more intensively in the state of Maranhão [18, 22]. Pilocarpine has important pharmaceutical properties. It is used to reduce the intraocular pressure in the treatment of glaucoma [14], as a stimulant of salivation and perspiration, and recently has been prescribed for the treatment of xerostomia, which a symptom refers abnormal dryness of the mouth due to the reduction of saliva production [5]. In spite of the importance of the plant and the pharmacological activity of pilocarpine, only a few reports have been published for this alkaloid in *Pilocarpus* [2, 3]. *Jaborandi* leaves are the only known source of pilocarpine, an imidazole alkaloid probably derived from histidine [6].

Several solvent-based extraction protocols for pilocarpine have been reported. Unfortunately, these methods often suffer from low extraction yields, long extraction times and potential existence of trace organic solvent in final products which decrease the product quality [25]. Several supercritical fluid and organic solvent-based extraction protocols to extract pilocarpine and other alkaloids have been reported [3, 19]. However, the use of organic solvents for
the recovery of natural products has several drawbacks, including safety hazards, high energy input, low product quality, environmental risk and toxicological effects [21]. Enzyme-based extraction of bioactive compounds from plants is a potential alternative to conventional solvent-based extraction methods. Enzymes are ideal catalysts to assist in the extraction, modification or synthesis of complex bioactive compounds of natural origin. Enzyme-based extraction is based on the inherent ability of enzymes to catalyze reactions with exquisite specificity, regioselectivity and an ability to function under mild processing conditions in aqueous solutions [9]. Thus, enzymes can degrade or disrupt cell walls and membranes efficiently enabling better release and extraction of bioactives [16]. This method also offers the possibility of greener chemistry as pressure mounts on the food industry and even pharmaceutical companies to identify cleaner routes for the extraction of new compounds [13].

Based on increase attention of enzyme-assisted extraction methods for their efficiency in extraction and eco-friendly aspect, we have designed and optimized extraction procedure for pilocarpine from P. jaborandi using Viscozyme® L as a catalytic enzyme. Viscozyme® L is a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase which can effectively break cell wall in plants. In this study, variables such as substrate concentration, reaction conditions (temperature, pH and reaction time) were optimized to obtain highest quantity of pilocarpine. To our knowledge, this is the first report utilizing enzyme as an alternative to acid-base method to extract the pilocarpine.

Materials and Methods

Materials, chemicals and instruments

P. jaborandi was imported from Brazil, South American. Viscozyme® L (KTN02118, Novozyme, Denmark), which contains a range of carbohydrases consisting of arabanase, β-glucanase, cellulase, hemicellulase and xylanase, was used in enzymatic hydrolysis. Pilocarpine standard and other chemical were obtained from Sigma Co (St. Louis, MO). All organic solvents and other chemicals were at the analytical grade from SK Korea Co. (Korea), except for high performance liquid chromatography (HPLC) grade (J.T Baker. USA). HPLC (LC-10A, Shimadzu, Co., Kyoto, Japan) associated with UV-visible detector (SPD-10A, Shimadzu, Co., Kyoto, Japan) were used for the determination of pilocarpine in P. jaborandi.

Viscozyme® L aided pilocarpine extraction from P. jaborandi

Twenty grams Pilocarpus leaves were grinded to make powder form. For the enzyme-aided hydrolysis reaction, 100 mg of leaves powder was taken and suspended in 40 ml of acetate buffer with different pH ranging from 3 to 6 while keeping all other variables including temperature, time (10 h) and enzyme concentration (1%). Once the optimum pH showing the highest yield of pilocarpine was determined, the other parameters were tested at the optimum pH. The next parameter tested was temperature, where temperature was the only variant from room temperature to 50°C while other variable were kept constant. Similarly, next variable tested was a time for extraction. Reaction was performed for various durations from 10 to 40 h to determine the time for maximum extraction yield. Once all the physical parameters for extraction were optimized, the final variable, enzyme concentration was determined. Different volume (ml) of Viscozyme® L solution was added to make final concentration of enzyme ranging from 1% to 12.5% (v/v). Once the reaction was complete, the residue was filtered. The solution containing pilocarpine was basified by 3 M sodium carbonate and extracted with chloroform. The chloroform was evaporated in speed vac. Extracted pilocarpine was dissolved in methanol and analyzed by HPLC.

HPLC and quantification

Pilocarpine in the extracts was analyzed by reversed phase HPLC. The alkaloid was separated in a Mightysil RP-18 GP column (150 × 4.6 mm ID., Kanto Chemical, Tokyo). Solution used for elution was 13.5 ml H₃PO₄ and 3 ml triethylamine in 850 ml MilliQ H₂O (pH 3 adjusted with NaOH) with further addition of 112 ml MeOH [7]. The flow rate was maintained at 1 ml/min and the detection was monitored with a UV monitor operating at 212 nm. A standard curve with different concentration of standard pilocarpine versus the peak area calculated from HPLC analysis was used for quantification of pilocarpine in crude extract. The concentrated crude extract was diluted to make sure that the area value lies within the standard curve value to avoid error. To confirm that the extracted compound was pilocarpine the compound was collected by preparative HPLC and proton NMR was conducted by Varian.