Korean J. Microbiol. Biotechnol. (2013), 41(2), 236–241
http://dx.doi.org/10.4014/kjmb.1303.03005
pISSN 1598-642X eISSN 2234-7305

KJMB
Korean Journal of Microbiology and Biotechnology

Enzymatic Extraction of Pilocarpine from *Pilocarpus jaborandi*

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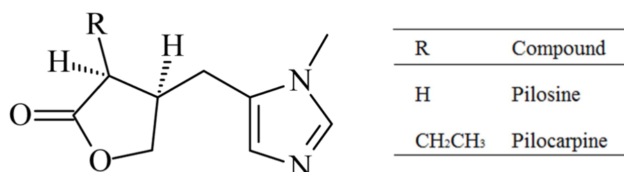
Received : March 25, 2013 / Revised : April 24, 2013 / Accepted : April 25, 2013

Pilocarpine is an imidazole alkaloid, found exclusively in the *Pilocarpus* genus, with huge pharmaceutical importance. In order to extract pilocarpine from *Pilocarpus jaborandi*, environmentally friendly enzyme-assisted extraction was applied. Viscozyme® L, a commercially available enzyme cocktail, was used for the study. The conditions for extraction were optimized on the basis of substrates, enzymes, temperatures and pHs. Optimum conditions for extraction with the highest yield were 30 h reaction of 100 mg substance at 45°C in 40 ml of 50 mM acetic acid, pH 4. A 10% enzyme concentration was found to be the best for extraction. Total pilocarpine content after extraction was analyzed by HPLC. The total pilocarpine content (1.14 µg/mg) obtained from Viscozyme® L treatment was 3.08-fold greater than those of the control treatment (0.37 µg/mg).

Keywords: Enzyme-assisted extraction, pilocarpine, *Pilocarpus jaborandi*, Viscozyme® L.

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].



R	Compound
H	Pilosine
CH_2CH_3	Pilocarpine

Fig. 1. Chemical structure of pilocarpine and its analogue pilosine.

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Several solvent-based extraction protocols for pilocarpine have been reported. Unfortunately, these methods often suffers 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 H_3PO_4 and 3 ml triethylamine in 850 ml MilliQ H_2O (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

Unity-Inova 300 MHz in CD_3OH.

Results and Discussion

Pilocarpine extraction

In general, the efficiency of the enzyme-aided phytochemical compounds extraction was influenced by multiple variables including the pH of reaction solution, the reaction temperature and time but not limited to enzyme type and concentration, and their effects were either independent or interactive [10, 12, 16, 20]. Preliminary studies were performed in order to determine the required enzyme concentration, pH and time for the extraction of the pilocarpine compounds from *Pilocarpus* leaves using acetate buffer of different pH and enzyme concentrations.

Prior to the optimization, the extracted compound collected in preparative HPLC was confirmed as pilocarpine from ^{1}H-NMR analysis (Fig. S1). The results show that the extraction yield of the pilocarpine compounds was dependent on the all tested variables. First, the effect of pH in reaction solution was evaluated using acetate buffer adjusted to different pH range (pH 2.0-5.0). As shown in Fig. 2A, pilocarpine extraction yield reached maximum level at pH 4.0, but subsequently decreased at higher pH. It has been known that pH of the reaction solution plays an important role in cell wall hydrolysis and polyphenol extraction in plants [1, 23, 26]. Furthermore, Viscozyme® L has been found to be stable at lower pH ranging from 4.0 to 5.0 [8]. Therefore, optimum pH of the reaction was optimized to pH 4.0.

The selection of an appropriate extraction temperature was the second step in the preliminary studies. The experiments were carried out ranging from room temperature to 50°C of the experimental design. As expected, the extraction yield of pilocarpine was increased along with temperature increases as shown in Fig. 2B. This might be due to the enhanced solubility and diffusion coefficient whereas reduced viscosity coefficient of pilocarpine compound at higher temperature [4, 11, 24]. Another plausible reason might be better enzyme activities at higher reaction temperature. Though, not many alkaloids have been extracted by enzymatic method but similar results has been reported by some researchers for polyphenols who found that a proper given temperature may help in extraction of antioxidant, while too high or low temperature had a significantly negative effect in case of polyphenol extraction [10, 15]. Beyond 45°C the extraction of pilocarpine decreases probably due to instability of enzyme at higher temperature (Fig. 2B).

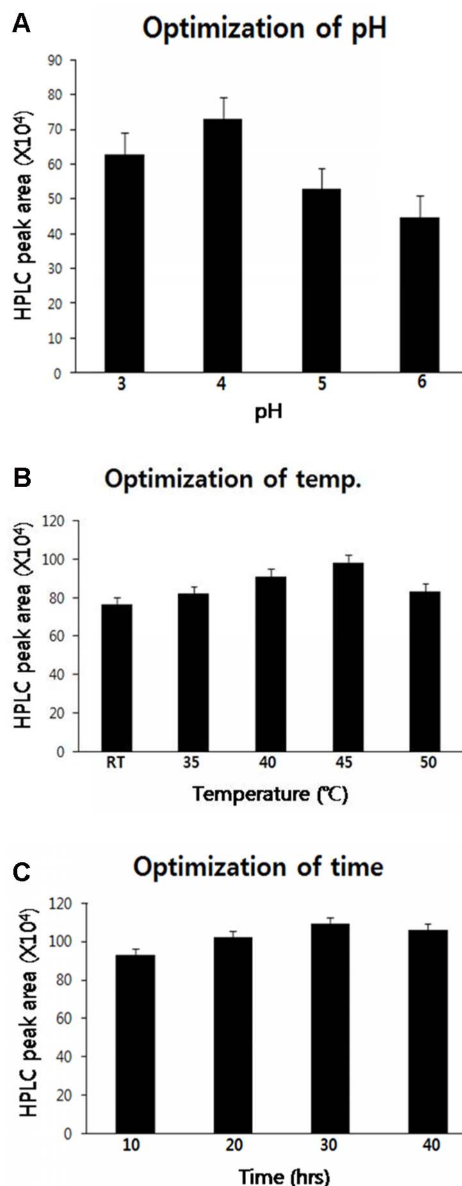

Fig. 2. (A) Graphical representation of pH-dependent extraction when all other variables were kept constant, temperature (RT), time 10 h and enzyme concentration (1%), (B) Graphical representation of temperature-dependent extraction at optimum pH 4, while other two parameters were constant, time 10 h and enzyme concentration (1%), (C) Graphical representation of time-dependent extraction at optimum pH 4, temperature (45°C) and enzyme concentration (1%).
All graphs were plotted between variable and area obtained by HPLC analysis. Each column represents the mean ± S.D. of triplicate determinations.

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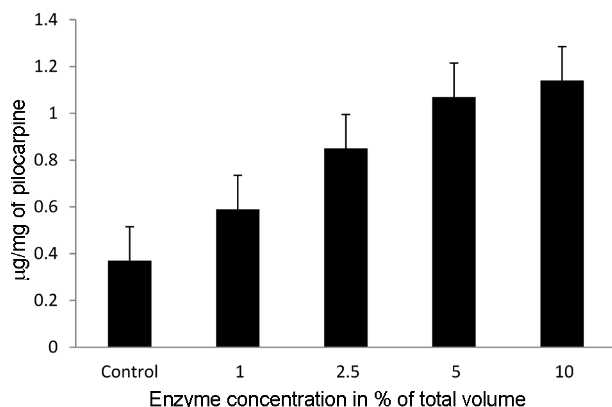

Fig. 3. Graphic representation of total pilocarpine contents in the optimized condition with varying enzyme concentration (1, 2.5, 5, and 10) in % of total volume. Control, no enzyme included.
Each column represents the mean ± S.D. of triplicate determinations. Control, 0.37 ± 0.15 ug/mg; 1.0%, 0.59 ± 0.14 ug/mg; 2.5%, 0.82 ± 0.14 ug/mg; 5.0%, 1.33 ± 0.15 ug/mg; 10.0%, 1.14 ± 0.15 ug/mg. A standard curve with different concentration of standard pilocarpine versus the peak area calculated from HPLC analysis was used for quantification of pilocarpine.

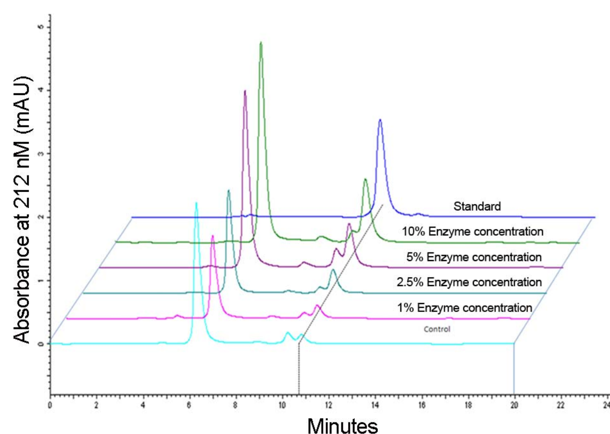

Fig. 4. HPLC chromatogram of pilocarpine contents in the optimized condition with varying enzyme concentration (1, 2.5, 5, and 10) in % of total volume.
Control, no enzyme included; Standard, pilocarpine chemical obtained from Sigma Co. The flow rate was maintained at 1 ml/min and the detection was monitored with a UV monitor operating at 212 nM.

Different incubation times for enzymatic reaction were also tested. The optimum reaction time was found to be 30 h and there was no further increase in pilocarpine content in longer reaction times (Fig. 2C). In the test to evaluate optimum enzyme concentration, the yield of pilocarpine increases with increasing enzyme concentration (Fig. 3). 10% of Viscozyme® L resulted in highest extraction yield of pilocarpine among the tested enzyme concentration indicating that higher concentration enzyme was able to hydrolyze more *Pilocarpus* leaves. The optimum condition for enzymatic reaction is summarized in Table 1.

Quantification of pilocarpine

The quantity of extracted pilocarpine at optimum condition was compared with one of the control sample of which same extraction procedure was conducted without enzyme. A standard curve obtained from area as function of different concentrations (20-360 μg/ml) of pilocarpine in HPLC analysis was used to quantify the amount of extracted pilocarpine from the *Pilocarpus* leaves. The HPLC chromatograms of pilocarpine extracted at optimum condition with varying enzyme concentration were compared with control sample in Fig. 4. The level of total pilocarpine content obtained from 10% Viscozyme® L treatment was 3.08-folds greater than those of the control treatment (Fig. 3) reaching upto 1.14 μg/mg of *Pilocarpus* leaves.

Conclusions

Although several solvent extraction procedures have been designed for extraction of pilocarpine [3], no studies with respect to enzymatic extraction of pilocarpine has been carried out till date. In this paper, we have optimized the temperature, pH, enzyme concentration and reaction time for the extraction of pilocarpine using a Viscozyme® L. The amount of pilocarpine from enzyme extraction was found to be 3.08-fold higher than that of control sample. This is the first report of the enzymatic extraction of pilocarpine. 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]. The method is also more economic and environmental friendly com-

Table 1. Optimized condition for extraction of pilocarpine with respect to Viscozyme® L concentration, pH, temperature and reaction time.

Substrate quantity	Solvent, Acetate	Enzyme quantity	pH	Temperature	Reaction time
100 mg	40 ml	10%	4	45°C	30 h

pared to conventional method of extraction where large quantity of organic solvent has been used. The content of pilocarpine extracted via this method is as efficient as those from solvent extraction method, therefore making this process suitable for industrial application.

Acknowledgment

This study was supported by a 2011 research grant from Hanseo University (Project code: 111 Bow gun 06).

References

1. Ahn, S. C., M. S. Kim, S. Y. Lee, J. H. Kang, B. H. Kim, W. K. Oh, B. Y. Kim, and J. S. Ahn. 2005. Increase of bioactive flavonoid aglycone extractable from Korean citrus peel by carbohydrate-hydrolyzing enzymes. *Korean J. Microbiol. Biotechnol.* **33**: 288-294.
2. Andrade-Neto, M., P. H. Mendes, and E. R. Silveira. 1996. An imidazole alkaloid and other constituents from *Pilocarpus trachyllophus*. *Phytochemistry* **42**: 885-887.
3. Avancini, G., I. N. Abreu, M. D. Saldana, R. S. Mohamed, and P. Mazzafera. 2003. Induction of pilocarpine formation in jaborandi leaves by salicylic acid and methyljasmonate. *Phytochemistry* **63**: 171-175.
4. Cacace, J. E. and G. Mazza. 2003. Mass transfer process during extraction of phenolic compounds from milled berries. *J. Food Eng.* **59**: 379-389.
5. Davies, A. N. and K. D. Broadley. 2001. Beighton, Xerostomia in patients with advanced cancer. *J. Pain Symp. Manag.* **22**: 820-825.
6. Dewick, P. M. 1997. Medicinal natural products: A biosynthetic approach. John Wiley & Sons.
7. Fan, T. Y., G. M. Wall, K. Sternitzke, L. Bass, A. B. Morton, and E. Muegge. 1996. Improved high-performance liquid chromatographic determination of pilocarpine and its degradation products in ophthalmic solutions: importance of octadecylsilane column choice. *J. Chromatogr. A.* **740**: 289-295.
8. Fen, L. L., R. M. Illias, K. Kamaruddin, M. Y. Maskat, and O. Hassan. 2006. Development of rapid screening method for low-yielding chitosanase activity using remazol brilliant blue-chitosan as substrate. *Enzyme Microb. Technol.* **38**: 215-219.
9. Gardossi, L., P. B. Poulsen, A. Ballesteros, K. Hult, V. K. Svedas, D. Vasić-Racki, G. Carrea, A. Magnusson, A. Schmid, R. Wohlgemuth, and P. J. Halling. 2009. Guidelines for reporting of biocatalytic reactions. *Trends Biotechnol.* **28**: 171-180.
10. Landbo, A. K. and A. S. Meyer. 2001. Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (*Ribes nigrum*). *J. Agric. Food Chem.* **49**: 3169-3177.
11. Liyana-Pathirana, C. and F. Shahidi. 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem.* **93**: 47-56.
12. Meyer, A. S., S. M. Jepsen, and N. S. Sørensen. 1998. Enzymatic release of antioxidants for human low-density lipoprotein from grape pomace. *J. Agric. Food Chem.* **46**: 2439-2446.
13. Meyer, A. S. 2010. Enzyme technology for precision functional food ingredients processes. *Ann. N. Y. Acad. Sci.* **1190**: 126-132.
14. Migdal, C. 2000. Glaucoma medical treatment: philosophy, principles and practice. *Eye* **14**: 515-518.
15. Pinelo, M., B. Zornoza, and A. S. Meyer. 2008. Selective release of phenols from apple skin: mass transfer kinetics during solvent and enzyme-assisted extraction. *Sep. Purif. Technol.* **63**: 620-627.
16. Pinelo, M., A. Arnous, and A. S. Meyer. 2006. Upgrading of grape skins: signicance of plant cell-wall structural components and extraction techniques for phenol release. *Trends Food Sci. Technol.* **17**: 579-590.
17. Pinheiro, C. U. B. 1997. Jaborandi (*Pilocarpus* sp., Tutaceae): a wild species and its rapid transformation into a crop. *Econ. Bot.* **51**: 49-58.
18. Pinheiro, C. U. B. 2002. Extrativism, cultivation and privatization jaborandi (*Pilocarpus microphyllus* Stapf ex Holm.; Rutaceae) in Maranhão, Brasil. *Acta. Bot. Bras.* **16**: 141-150.
19. Saldana, M. D., R. S. Mohamed, M. G. Baer, and P. Mazzafera. 1999. Extraction of purine alkaloids from mat (*Ilex paraguariensis*) using supercritical CO_2. *J. Agric. Food Chem.* **47**: 3804-3808.
20. Sørensen, H. R., S. Pedersen, V. N. Anders, and A. S. Meyer. 2005. Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in an industrial ethanol fermentation residue. *Enzyme Microb. Technol.* **36**: 773-784.
21. Teo, C. C., S. N. Tan, J. W. Yong, C. S. Hew, and E. S. Ong. 2010. Pressurized hot water extraction (PHWE). *J. Chromatogr. A.* **1217**: 2484-2494.
22. Vieira, R. F. 1999. Conservation of medicinal and aromatic plants in Brazil. In: Janick, J. (Ed.), Perspectives on New Crops and New Uses, pp. 152-159. ASHS Press. Alexandria.
23. Weinberg, Z. G., B. Akiri, E. Potoyevski, and J. Kanner. 1999. Enhancement of polyphenol recovery from rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) by enzyme-assisted ensiling (ENLAC). *J. Agric. Food Chem.* **47**: 2959-2962.
24. Wettasinghe, M. and F. Shahidi. 1999. Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago ofcinalis* L.) seeds. *Food Chem.* **67**: 399-414.
25. Yang, B., Y. Jiang, J. Shi, F. Chen, and M. Ashraf. 2011. Extraction and pharmacological properties of bioactive compounds from longan (*Dimocarpus longan* Lour.) fruit-a review. *Food Res. Int.* **44**: 1837-1842.
26. Zheng, H. Z., H. R. Lee, S. H. Lee, C. S. Kim, and S. K. Chung. 2008. Pectinase assisted extraction of polyphenol from apple pomace. *Chin. J. Anal. Chem.* **36**: 306-310.

http://dx.doi.org/10.4014/kjmb.1303.03005

국문초록

***Pilocarpus jaborandi*로부터 필로카르핀의 효소반응추출.** 조전호[1], 사우라브 바타라이[1], 오태진[1]*, 장종화[2]*. [1]*선문대학교 제약공학과*, [2]*한서대학교 치위생학과*

필로카르핀은 *Pilocarpus* 속으로부터 유일하게 분리되는 이미다졸계 알칼로이드로서 상당히 제약적으로 중요하다. *Pilocarpus jaborandi*로부터 필로카르핀을 추출하기 위하여 환경친화적인 효소를 이용한 추출법을 이용하였다. 본 연구에서는 상업적으로 이용할 수 있는 효소칵테일인 Viscozyme® L을 사용하였다. 추출 조건은 기질, 효소, 온도 및 pH 등에 기초하여 최적화되었다. 가장 높은 수율을 위한 최적화 조건은 pH4인 50 mM 아세트산 40 ml 하에서 45°C, 100 mg 기질, 30시간 반응이였다. 최적의 추출 효소농도는 10%이였다. Viscozyme® L 처리로부터 얻어진 전체 필로카르핀 함유량(1.14 μg/mg) 수준은 기존 처리방법에서 얻어지는 양(0.37 μg/mg)보다 3.08배 높은 것을 확인하였다.