嘉南藥理科技大學專題研究計畫成果報告

以生物分離法來製備 Cis-cinnamic acid

Bioseparation of trans-, cis-cinnamic acids by resting cell suspension of *Rhodotorula rubra* Y-1529

計畫編號：CNHN-89-01
執行期間：88 年 9 月 1 日至 89 年 6 月 30 日
計畫類別：■個別型　□整合型：
主持人：　王瑞顯　　計畫總主持人：
協同研究：孫芳明　　協同研究：

ABSTRACT

A resting cell suspension of *Rhodotorula rubra* Y-1529 has demonstrated has the capability to separate racemic mixture of trans-, cis-cinnamic acids on a mg scale. The stereoselectivity of these bioconversion processes have been estabilished. The procedure therefore represents a new and valid method for the convenient method of preparation of pure cis-cinnamic acid and 2-chlorocinnamic acid.

Keywords: *Rhodotorula rubra*, cis-cinnamic acid, racemic mixture, bioconversion.

INTRODUCTION

Stereochemistry at the molecular level plays a dominant role in biological activity since most of the cellular molecular compounds are stereoisomers. There are significant differences in the activities of bioactive enantiomers/diastereomers produced by or active against living microorganisms which are vitally important to the pharmaceutical, flavor and food industries, and in the bioremediation of organic pollutants (Aheldon 1993).

Both trans- and cis-cinnamic acids (3-phenyl-2-propenic acid) are presented in nature (Wolfrram et al. 1994). In plant cells, trans-cinnamic acid is an anti-auxin compound and also an important precursor and intermediate for synthesis of lignin, flavornoids, and phenolic compounds (Floss 1977). For microorganism, it can used as a carbon source (Coulson et al. 1959; Kawakami 1980), converted to flavor compound (Hilton and Cain 1990), or to other secondary metabolites (Arnone 1993). The cis-cinnamic acid may play an important role in the physiology of plant cells and fungi (Hess et al. 1975). Because of the unavailable of pure and stable cis-cinnamic acid (Lindenfors 1957). The role for cis-cinnamic acid on nutritional, toxicological, and metabolic pathway in plant cells and microorganism are unknown. To date, the trans- and cis-cinnamic acid were separated by chromatography methods. The chromatography methods are expensive, laborious and time consuming processes. Moreover, it is almost impossible to achieve a 100% separation of these two stereoisomers and the presence of impurity in the cis-isomers may cause unstability of these compounds (Lindenfors 1958).

Bioconversion/biotransformation is defined as the use of microbial cells to perform specific modification or interconversion of chemical structure; and most importantly it is a stereospecific process which make it become an useful tool for the production or separation of pure stereoisomers (Bestetti et al. 1994, 1995). In this paper, we will introduce a new, fast, and cheap method-using

stereospecific bioconversion systems to separate trans-, cis-cinnamic acids and chlorinated cinnamic acids by a resting cell suspension of *Rhodotorula rubra* Y-1529.

MATERIALS AND METHODS

Chemicals:

trans-Cinnamic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI) with the purities of 95-99.5%. The cis-isomers of cinnamic acid and chlorinated cinnamic acids were prepared following the method developed by Sun and Traxler (1998). All other chemicals were Baker Reagent (Phillipsburg, NJ) or Fisher Certified ACS (Fair Lawn, NJ). All solvents were obtained from Fisher Scientific Co. (Agawam, MA) and are HPLC or GC grade.

Organism and culture conditions:

Rhodotorula rubra Y-1592 was obtained from the Northern Regional Laboratory (Peoria, Il), checked for purity by colony morphology, typical orange color, Gram stain; and grown for G-1 stock cultures in YEPD medium (2 g D(+)-glucose, 2 g Bacto Peptone, 1 g Yeast Extract, 100 ml deionized water) to the point of late exponential growth phase (OD = 0.6) at 28^0C with agitation at 200 rpm. The cells was then centrifuged at 3000 X G for 15 min, washed twice by centrifugation using 10mM Phosphate Buffer at pH 7.0 and after the final wash the cells were resuspended in 3 mM Pipes buffer containing 0.02% $MgSO_4$ at pH 7.5.

Bioseparation System:

The reaction mixtures consisted of 10, 20, and 40 mg of the racemic mixtures of cinnamic acids and chlorinated cinnamic acids dissolved in absolute ethanol added to 50 ml of Pipes buffer, pH 7.5. The ethanol was removed by sparging with a nitrogen gas, and the resting cell suspension added to a final concentration of 2.69*0.16 mg/ml (OD=1.4). Ten ml samples were taken from each flask at each time interval, clarified by centrifugation at 3000 X G for 30 minutes, filtered through 0.2 μm polycarbonate membrane filters (Proetics, Livermore, CA) and held at -20^0C until analysis.

Analysis of Substrates and Products:

The trans-isomers of cinnamic acid and bioconversion products were separated and quantified by HPLC coupled with a photodiode array UV-Visible detection. The Waters system (Milford, MA) consisted of a fluid pump Model 600E, a Model 991 photodiode array detector and fitted with a Waters Nova-pak C_{18} reverse phase analytical column (3.9mm ID X 150mm). The mobile phase was 52 methanol : 48 deionized water (containing 0.5% acetic acid), at a flow rate of 1ml/min. The injection volume was 10 μl. The spectra were scanned continuously between 190 and 400 nm. The chromatographs were extracted at 270 nm for quantification. Data acquisition and peak integration were performed by transferring the data to an Dell 133P PC using the Millennium 2010 chromatography manager program.

RESULTS AND DISCUSSION

Bioseparation of racemic mixture of cinnamic acids:

The HPLC chromatograms of bioconversion of 10 mg of cis- and trans-mixtures of cinnamic acids by resting cell suspensions of *R. rubra* Y-1529 (cell dry wt. 2.69±0.13 mg/ml) in 50 ml of Pipes buffer (3mM, pH 7.5) at 28^0C, 200 rpm. After 3.5 hr only one peak was left and remained unchanged for 120 hr. When the peak was examined by using the Millennium 2010 MS

window, it was found that the peak was a one-component peak and that the one-component is cis-cinnamic acid. After extraction with 50ml of ethyl acetate twice and evaporation of the ethyl acetate by rotatory evaporator with a 20psi vacuum at 65^{0}C, a pure cis-cinnamic acid was obtained, with a recovery of 62%. When the substrate concentration was increased from 10 mg to 40 mg, all of the trans-cinnamic acid was disappeared within 3.5 hr and only one peak left while the cis-cinnamic acid remained unchanged (Table 1).

Table 1: Quantification data of trans-, and cis-cinnamic acid for the bioseparation of racemic mixtures of cinnamic acid by *R. rubra* Y-1529 at cell mass level of 2.69±0.16 g/L in 50ml Pipes buffer (3mM), pH 7.5 under 28^{0}C, 200 rpm

Time (hour)/substrate concentration (mmole)	trans-cinnamic acid (0.034 mmole = 5mg/50ml)	cis-cinnamic acid(0.034 mmole = 5mg/50ml)	trans-cinnamic acid (0.068 mmole = 10mg/50ml)	cis-cinnamic acid(0.068 mmole = 10mg/50ml)	trans-cinnamic acid (0.134 mmole = 20mg/50ml)	cis-cinnamic acid(0.134 mmole = 20mg/50ml)
0	0.040	0.033	0.070	0.062	0.138	0.131
1	0.011	0.033	0.025	0.065	0.059	0.133
2	0	0.032	0	0.063	0.011	0.131
3.5	0	0.032	0	0.062	0	0.133

For the bioseparation of racemic mixture of cis- and trans-cinnamic acid by *R. rubra*, up to 20mg of cis-cinnamic acid can successfully be separated from racemic mixture of cinnamic acid within 3.5hr by *R. rubra* at a cell mass level of 2.69±0.16g (dry wt.)/L. From the bioseparation experiments of trans-, cis-cinnamic acid, the concentration for the cis-isomers remained unchanged after 120 hr. This indicates *R. rubra* only biodegrades trans-cinnamic acid, but not the cis-isomers. These results tell us that the enzyme(s) which catalyze the modification reaction of the unsaturated double bond on the three carbon side chain is specific for the trans-configuration (stereospecific). This mechanism is similar to the β-oxidation of unsaturated fatty acids. In β-oxidation of unsaturated fatty acids, after the enoyl CoA ester is formed, the next step is the hydroxylation of the unsaturated double bond between C-2 and C-3 by enoyl CoA hydratase. This enzyme is specific for trans-configuration of the double bond. Therefore, unsaturated fatty acid with cis-configuration double bond on its structure cannot be metabilized by β-oxidation, unless the cis-configuration double bond is transformed to trans-configuration by enoyl CoA isomerase before the hydration step (Schulz 1991). In *R. rubra*, this isomerase is either absent or because of the totally different chemical and physical properties of cis-isomers of cinnamic acid and chlorinated cinnamic acids (when compared with the trans-isomers) it cannot work on cis-cinnamic acid to convert cis-isomers to trans-isomers. Therefore, the cis-isomers of cinnamic acid remained unchanged for 120 hours and cannot be bioconverted by *R. rubra*.

The results from this study suggest *R. rubra* can be used as a tool to separate trans- and cis-isomers of cinnamic acid to yield pure cis-isomers. When compare with the traditional chromatography methods, this bioseparation process is cheaper, faster, less environmental hazard and should be easy to scale up.

References:

Aheldon R. 1993. Chirotechnology: industrial synthesis of optical active compounds. Marcel Dekker, Inc., New York. pp 7-20.

Arnone A., R. Cardillo, G. Nasini, and O. V. De Pava. 1993. Two cinnamic acid allenic ethers from the fungus *Clitocybe eucalyptorum*. Phytochem. 32: 1279-1281.

Bestetti G., P. Di Gennaro, E. Galli, B. Leoni, F. Felizzoni, G. Sello, and D. Bianchi.1994. Bioconversion of substituted naphthalenes to corresponding salicylic acid. Appl. Microbiol. Biotechnol. 40:791-793.

Bestetti G., D. Banchi, A. Bosetti, P. Di Gennaro, E. Galli, B. Leoni, F. Pelizzoni, and G. Sello. 1995. Bioconversion of substituted naphthalenes to the corresponding 1,2-dihydro-1,2-dihydroxy derivatives, Determination of the regio- and stereochemistry of the oxidation reactions. Appl. Microbiol. Biotechnol. 44:306-313.

Campbell I. M., M. A. Gallo, C. A. Jones, P. R. La Sitis, and L. M. Rosato. 1987. Role of cinnamate in benzoate production in *Penicillium brevicopactum*. Phytochem. 26: 1413-1415.

Coulsion C. B., and W. C. Evans. 1959. Microbial degradation of trans-cinnamic acid by Pseudomonas. Chem. Ind. 17:543-544.

Floss H. G. 1979. The Shkimate pathway. In Biochemistry of plant phenolics. T. Swain, J.B. Harborne, and C. F. Van Sumeze(eds). Plenum Press. New York.

Hess S. L., P. J. Allen, D. Nelson, and H. Lester. 1975. Mode of action of methyl cis-ferulate, the self-inhibitor of stem rust uredospore germination. Physiol. Plant Pathway. 5:107-112.

Hilton M. D. and Cain W. J. 1990. Bioconversion of cinnamic acid to acetophenone by a Pseudomonad: Microbial production of a natural flavor compound. Appl. Environ. Microbiol. 56:623-627.

Kawakami, H. 1980. Degradation of lignin-related aromatics and lignins by several pseudomonads, p. 103-125. T. K. Kirk; T. Higuchi; and H> M. Chang (ed.), Lignin biodegradation: microbiology, chemistry, and potential applications, vol. 2. CRC Press, Inc., Boca Raton, Fla.

Lindenfors S. 1957. Separation of cis-trans isomers by ion-exchange chromatography: 4chloro-cis-cinnamic acid and 2,4-dichloro-cis-cinnamic acid. Arkiv Kemi. 10:561-568.

Lindenfors S. 1958. On the trans-cis isomerization of cinnamic acids by the action of ultraviolet rays: 3-chloro-cis-cinnamic acid and 3,5-dichloro-cis-cinnamic acid. Arkiv Kemi. 12:267-279.

Schulz H. 1991. Oxidation of fatty acid. In: Biochemistry of lipids, Lipoproteins and membranes. D. E. Vance and J. Vance (eds). Elsevier Science Publishers. Netherland. pp 87-101.

Sun F. M. and R. W. Traxler. 1998. Bioconversion of cis-, trans-cinnamic acids and chlorinated cinnamic acids by resting cell culture of Rhodotorula rubra Y-1529. Ph.D. dissertation of Uni. of Rhode Island.

Wolfrram S., T. Weber, B. Grenacher, and E. Scharrer. 1994. A Na+-dependent mechanism is involved in mucosal uptake of cinnamic acid across the jeiunal brush border in rats. Nutrient metabol. 113:1300-1308.