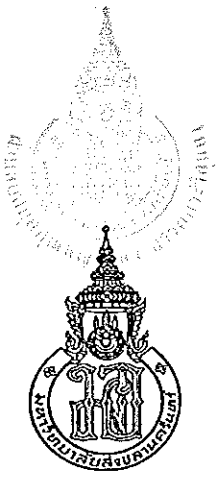

Screening, Isolation, Purification of Hydroxynitrile Lyase from Plants and Its Application

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

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Thesis Title	Screening, Isolation, Purification of Hydroxynitrile Lyase from Plants and Its Application
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ชื่อวิทยานิพนธ์	การคัดเลือก การแยก การทำบริสุทธิ์เอนไซม์ไฮดรอกซีไนตริลไลเอสจากพืช และการประยุกต์ใช้
ผู้เขียน	นางสาวเตชวรี เอื้อตรงจิตต์
สาขาวิชา	เทคโนโลยีชีวภาพ
ปีการศึกษา	2550

บทคัดย่อ

การคัดเลือกพืชที่มีการสร้างไซยาไนด์ (HCN) จากส่วนใบ, เมล็ด, ผล และหน่อของพืช จำนวน 176 ชนิด จาก 26 ตระกูล ของพืชในประเทศไทยโดยวิธีการทดสอบด้วยกระดาษพิเครท (picrate paper) พบพืชที่สามารถสร้างไซยาไนด์ได้ที่รายงานแล้วจำนวน 11 ชนิด และมีปริมาณไซยาไนด์ที่ปลดปล่อยจากพืช ดังนี้ ใบของผักหนาม (*Lisia spinosa*; 1,258 ส่วนในล้านส่วน), ใบของตำแยแมว (*Acalypha indica*; 1,020 ส่วนในล้านส่วน), ใบของต้นประ (*Elateriospermum tapos*; 7 ส่วนในล้านส่วน), ใบของต้นยางพารา (*Hevea brasiliensis*; 2,961 ส่วนในล้านส่วน), ใบของต้นมันสำปะหลัง (*Manihot esculenta*; 1,013 ส่วนในล้านส่วน), หน่ออ่อนของต้นไผ่ป่า (*Bambusa bambos*; 1,822 ส่วนในล้านส่วน), ใบของกระถินเทศ (*Acacia farnesiana*; 4 ส่วนในล้านส่วน), ใบของต้นประดู่ลาย (*Dalbergia cochinchinensis*; 1,795 ส่วนในล้านส่วน), ใบของต้นสรีมาลา (*Passiflora cocinea*; 833 ส่วนในล้านส่วน), ใบของต้นเสาวรส (*Passiflora edulis*; 1,085 ส่วนในล้านส่วน), และใบของต้นสุดนธรส (*Passiflora quadrangularis*; 1,169 ส่วนในล้านส่วน) และตรวจพบพืชที่สร้างไซยาไนด์ได้ในศึกษาครั้งนี้เป็นครั้งแรก จำนวน 5 ชนิด และปริมาณไซยาไนด์ที่ปลดปล่อยจากพืช ได้แก่ ใบของต้นไม้มะค่า (*Afzelia xylocarpus*; 1,329 ส่วนในล้านส่วน), เมล็ดที่กำลังงอกของลูกเนียงเพาะ (*Archidendron jiringa*; 17 ส่วนในล้านส่วน), ใบของต้นประดู่บ้าน (*Dalbergia floribunda,* 625 ส่วนในล้านส่วน), ใบของต้นกระดาดดำ (*Xanthosoma nigrum*; 15 ส่วนในล้านส่วน), และใบของต้นกระเบากลัก (*Hydnocarpus ilicifolia*; 707 ส่วนในล้านส่วน) พีเอชและอุณหภูมิมีผลต่อการสลายตัวของสารประกอบไซยาโนไฮดรินแมนเดโลไนตริล กิจกรรมของเอนไซม์ (*S*)-ไฮดรอกซีไนตริลไลเอส ถูกค้นพบในใบของต้นยางพารา (*Hevea brasiliensis*) ปริมาณ 3.28 ยูนิต/มิลลิกรัมโปรตีน และในใบของต้นมันสำปะหลัง (*Manihot esculenta*) ปริมาณ 2.13 ยูนิต/มิลลิกรัมโปรตีน ขณะที่ใบ, เมล็ด และเปลือกของต้นเสาวรส (*Passiflora edulis*) มีกิจกรรมของเอนไซม์ (*R*)-ไฮดรอกซีไนตริลไลเอส ปริมาณ 1.47, 1.23, และ

0.92 ยูนิต/มิลลิกรัมโปรตีน และสามารถสังเคราะห์สารประกอบไซยาโนไฮดริน โดยให้ค่า อิแนนทิโอเมอริกแอกเซส 54.6, 47.9 และ 33.2 เปอร์เซ็นต์ ตามลำดับ

การสังเคราะห์แบบอสมมาตรของสารประกอบ (*R*)-แมนเดโลไนตริลในระบบสองสถานะโดยใช้เอนไซม์ไฮดรอกซีไนตริลไลเอสจากเสาวรสเป็นครั้งแรก พบว่า ปัจจัยหลายอย่างมีผลต่อค่าความบริสุทธิ์ของอิแนนทิโอเมอร์ และค่าความเร็วเริ่มต้นของปฏิกิริยา พีเอชและอุณหภูมิเป็นปัจจัยสำคัญในการควบคุมความบริสุทธิ์ของอิแนนทิโอเมอร์ของผลิตภัณฑ์ ค่าพีเอชและอุณหภูมิที่เหมาะสม ได้แก่ พีเอช 4 และ อุณหภูมิ 10 องศาเซลเซียส ตามลำดับ ณ สภาวะที่เหมาะสมนี้ ปฏิกิริยาเดมีที่เกิดขึ้นเองโดยไม่ได้เกิดจากปฏิกิริยาของเอนไซม์ซึ่งก่อให้เกิดสารผสมราเซเมทของแมนเดโลไนตริลถูกยับยั้งแทบทั้งหมด เอนไซม์ไฮดรอกซีไนตริลไลเอสจากเสาวรสมีกิจกรรมคงเหลือมากกว่า 80% เมื่อบ่มเอนไซม์เป็นเวลา 12 ชั่วโมง ในระบบที่มีเมทิลเทอร์เทียรี่บิวทิวอีเทอร์, ไดบิวทิลอีเทอร์, เฮกเซน และไดไอโซโพรพิลอีเทอร์เป็นตัวทำละลายอินทรีย์ ขณะที่ไดเอทิลอีเทอร์ และเอทิลอะซิเตทเป็นตัวทำละลายอินทรีย์ที่ไม่เหมาะสม ชนิดของตัวทำละลายอินทรีย์มีผลต่อค่าความเร็วเริ่มต้นของปฏิกิริยาแตกต่างกัน เมื่อใช้ระบบตัวทำละลายอินทรีย์ที่มีค่า log *P* น้อยกว่า 3.5 จะให้ค่าอิแนนทิโอเมอริกแอกเซสของผลิตภัณฑ์สูง ระบบสองสถานะของไดบิวทิลอีเทอร์ที่มีน้ำ 30 เปอร์เซ็นต์โดยปริมาตรให้ค่าความเร็วเริ่มต้นของปฏิกิริยาและค่าความบริสุทธิ์ของ (*R*)-แมนเดโลไนตริลสูงที่สุด ค่าความเข้มข้นที่เหมาะสมสำหรับเบนซัลดีไฮด์และอะซิโตนไซยาโนไฮดริน คือ 250 และ 900 มิลลิโมลาร์ ตามลำดับ ขณะที่ค่าความเข้มข้นของเอนไซม์ที่เหมาะสมคือ 26.7 ยูนิต/มิลลิลิตร สารประกอบ (*R*)-แมนเดโลไนตริลที่มีค่าความบริสุทธิ์ของอิแนนทิโอเมอร์สูงถูกสังเคราะห์ขึ้นภายใต้สภาวะที่เหมาะสม ได้ปริมาณผลผลิต และค่าอิแนนทิโอเมอริกแอกเซสของผลิตภัณฑ์ 31.6 และ 98.6 เปอร์เซ็นต์ ตามลำดับ เอนไซม์ที่อยู่ในส่วนของน้ำสามารถนำมาใช้ซ้ำได้ 4 ครั้ง โดยยังคงให้ค่าความบริสุทธิ์ของ อิแนนทิโอเมอร์สูง

เอนไซม์ไฮดรอกซีไนตริลไลเอสจากเมล็ดโลควอท (*Eriobotrya japonica*) ถูกแยกและทำบริสุทธิ์ ได้ผลผลิตสุดท้าย 36 เปอร์เซ็นต์ และมีความบริสุทธิ์ 50 เท่า หลังจากผ่านกระบวนการแยกโดยการแยกส่วนตะกอนโปรตีนด้วยสารประกอบแอมโมเนียมซัลเฟตอิ่มตัวที่ 30-80 เปอร์เซ็นต์, การแยกโดยวิธีการโครมาโทกราฟีแบบคอลัมน์ โดยใช้เรซินแลกเปลี่ยนประจุลบดีอีเอดี (DEAE-Toyopearl) และเรซินจับจำเพาะคอนคานาวาลิน เอ (Concanavalin A Sepharose 4B) ซึ่งแสดงว่าเอนไซม์อาจมีส่วนของคาร์โบไฮเดรทเป็นองค์ประกอบ เอนไซม์บริสุทธิ์เป็นโมโนเมอร์มีน้ำหนัก 72 กิโลดาลตันเมื่อวิเคราะห์โดยวิธีการเจลฟิลเทรชัน และมีน้ำหนัก 62.3 กิโลดาลตันเมื่อวิเคราะห์โดยวิธีการอิเลคโทรโฟเรซิสโดยใช้เจล SDS เอนไซม์เป็นฟลาโวโปรตีนซึ่งมีหมู่ FAD เป็นหมู่โพรสเตทิก และแสดงค่า K_m 161 ไมโครโมลาร์ และค่าคงที่จำเพาะ (k_{cat}/K_m) 348 ต่อวินาที

ต่อมิลลิโมลาร์สำหรับแมนเดโลไนตริล ค่าพีเอชและอุณหภูมิที่เหมาะสม ได้แก่ พีเอช 5.5 และอุณหภูมิ 40 องศาเซลเซียส ตามลำดับ เอนไซม์มีความเสถียรต่อพีเอชและอุณหภูมิ อิออนของโลหะไม่จำเป็นต่อกิจกรรมของเอนไซม์ ขณะที่กิจกรรมของเอนไซม์ถูกยับยั้งอย่างมีนัยสำคัญโดยสารประกอบคอปเปอร์ซัลเฟต, เมอร์คิวริคลอไรด์, ซิลเวอร์ไนเตรท, เฟอร์ริคคลอไรด์, เบต้า-เมอร์แคบโทเอทานอล, ไอโอโดอะซิติกแอซิด, ฟีนิลเมทิลซัลโฟนิลฟูลโอไรด์ และไดเอทิลไพโรคาร์บอเนท ค่าคงที่จำเพาะของเอนไซม์ถูกวิเคราะห์เป็นครั้งแรกโดยใช้สารประกอบแอลดีไฮด์หลายชนิดเป็นสับสเตรท พบว่า เอนไซม์สามารถใช้ทั้งสารประกอบอโรมาติกและอลิฟาติกแอลดีไฮด์เป็นสับสเตรทได้ และแสดงความชอบต่อสับสเตรทขนาดเล็กมากกว่าสับสเตรทขนาดใหญ่

สารประกอบ (*R*)-แมนเดโลไนตริล ถูกสังเคราะห์โดยปฏิกิริยาการเคลื่อนย้ายหมู่ไซยาโน (transcyanation) ระหว่างสารประกอบแอลดีไฮด์และสารประกอบอะซิโตนไซยาโนไฮดริน โดยเอนไซม์ไฮดรอกซีไนตริลไลเอสจากเมล็ดโลควอท ในระบบสองสถานะระหว่างน้ำและตัวทำละลายอินทรีย์ พบว่า ค่าพีเอชและอุณหภูมิที่เหมาะสมสำหรับการสังเคราะห์สารให้ได้ค่าอิแนนทิโอเมอริกแอกเซสสูง ได้แก่ พีเอช 4.0 และอุณหภูมิ 10 องศาเซลเซียส เอนไซม์แสดงความคงตัวต่อตัวทำละลายอินทรีย์ซึ่งได้แก่ เอทิลอะซิเตท, ไดเอทิลอีเทอร์, เมทิลเทอร์เทียรี่บิวทิวอีเทอร์, ไดไอโซโพรพิลอีเทอร์, ไดบิวทิลอีเทอร์, และเฮกเซน เป็นเวลา 12 ชั่วโมง ตัวทำละลายอินทรีย์ที่ดีที่สุดที่ให้ค่าความเร็วเริ่มต้นของปฏิกิริยาและค่าอิแนนทิโอเมอริกแอกเซสสูง ได้แก่ ไดเอทิลอีเทอร์ อัตราส่วนของส่วนน้ำที่เหมาะสม คือ 50 เปอร์เซ็นต์โดยปริมาตร การสังเคราะห์สารประกอบ (*R*)-แมนเดโลไนตริลภายใต้สภาวะที่เหมาะสมพบว่า ปริมาณผลผลิตและค่าอิแนนทิโอเมอริกแอกเซสของ (*R*)-แมนเดโลไนตริลเมื่อสังเคราะห์เป็นเวลา 3 ชั่วโมง ได้แก่ 40 และ 99 เปอร์เซ็นต์ ตามลำดับ ส่วนน้ำที่มีเอนไซม์เป็นองค์ประกอบสามารถนำมาใช้ซ้ำได้ 4 ครั้ง

Thesis Title	Screening, Isolation, Purification of Hydroxynitrile Lyase from Plants and Its Application
Author	Miss Techawaree Ueatrongchit
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ABSTRACT

The leaves, seeds, fruits and shoots of 176 plant species in 26 families of Thailand were screened for the presence of cyanide (HCN) using the picrate paper test. The known cyanogenic plants and their cyanide contents were observed in 11 species which were leaves of *Lisia spinosa* (1,258 ppm), leaves of *Acalypha indica* (1,020 ppm), leaves of *Elateriospermum tapos* (7 ppm), leaves of *Hevea brasiliensis* (2,961 ppm), leaves of *Manihot esculenta* (1,013 ppm), young shoot of *Bambusa bambos* (1,822 ppm), leaves of *Acacia farnesiana* (4 ppm), leaves of *Dalbergia cochinchinensis* (1,795 ppm), young leaves of *Passiflora cocinea* (833 ppm), leaves of *Passiflora edulis* (1,085 ppm), and leaves of *Passiflora quadrangularis* (1,169 ppm). The new sources of cyanogenic plants and their cyanide contents were investigated in 5 species which were leaves of *Afzelia xylocarpus* (1,329 ppm), seedlings of *Archidendron jiringa* (17 ppm), leaves of *Dalbergia floribunda* (625 ppm), leaves of *Xantosoma nigrum* (15 ppm), and leaves of *Hydnocarpus ilicifolia* (707 ppm). The increase in pH and temperature accelerated the decomposition of the cyanohydrin (mandelonitrile) significantly. The (*S*)-hydroxynitrile lyases (HNLs) activity presented in leaves of *Hevea brasiliensis* (3.28 U/mg) and *Manihot esculenta* (2.13 U/mg), while (*R*)-HNL activity was found in leaves, seeds, and rind of *Passiflora edulis* with specific activity of 1.47, 1.23, and 0.92 U/mg, respectively. The (*R*)-HNL from leaves, seeds, and rind of *P. edulis* showed ability in cyanohydrin synthesis with enantiomeric excess of 54.6, 47.9, and 33.2%, respectively.

Asymmetric synthesis of (*R*)-mandelonitrile in biphasic system employing a hydroxynitrile lyase from *P. edulis* (*Pe*HNL) was reported for the first time. Several parameters influenced the enantiomeric purity of product and initial velocity of the reaction. Both pH and temperature were important parameters to control the enantiomeric purity of the product. The

optimum pH and temperature were pH 4 and 10 °C, respectively. At the optimum conditions, the spontaneous non-enzymatic reaction yielding the racemic of mandelonitrile was almost suppressed. The *Pe*HNL performed more than 80% residual activity in the solvent system of methyl-t-butyl ether, dibutyl ether (DBE), hexane, and diisopropyl ether while diethyl ether and ethyl acetate were not suitable solvents. The initial velocity was markedly affected by type of organic solvents in biphasic system. When using the organic solvent with log *P* lower than 3.5, high enantiomeric purity was obtained. The highest initial velocity of reaction and enantiomeric purity of (*R*)-mandelonitrile were obtained in biphasic system of DBE with the aqueous phase content of 30% (v/v). The optimum substrate concentrations were 250 mM for benzaldehyde and 900 mM for acetone cyanohydrin, while the optimum enzyme concentration was 26.7 units/ml. The high enantiomeric purity of (*R*)-mandelonitrile was successfully obtained with 31.6% conversion and enantiomeric excess of 98.6%. The enzyme could be used for four batch reactions with high enantiomeric purity of product.

A hydroxynitrile lyase has been isolated and purified to homogeneity from seeds of *Eriobotrya japonica* (loquat). The final yield of 36% with 50-fold purification was obtained by 30-80% $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-Toyopearl and Concanavalin A Sepharose 4B, which suggested the presence of a carbohydrate side chain. The purified enzyme is a monomer with a molecular mass of 72 kDa as determined by gel filtration and 62.3 kDa as determined by SDS-gel electrophoresis. The enzyme is a flavoprotein containing FAD as a prosthetic group and exhibits a K_m of 161 μM and k_{cat}/K_m of 348 $sec^{-1}mM^{-1}$ for mandelonitrile. The optimum pH and temperature were pH 5.5 and 40 °C, respectively. The enzyme showed excellent stability with regard to pH (pH 3-9) and temperature (0-60 °C). Metal ions were not required for its activity, while the activity was significantly inhibited by $CuSO_4$, $HgCl_2$, $AgNO_3$, $FeCl_3$, β-mercaptoethanol, iodoacetic acid, phenylmethylsulfonylfluoride, and diethylpyrocarbonate. The specificity constant (k_{cat}/K_m) of the enzyme was investigated for the first time using various aldehydes as substrates. The enzyme was active toward aromatic and aliphatic aldehydes and showed a preference for smaller substrates over bulky substrates.

(*R*)-Mandelonitrile was sucessfully synthesized by an enzymatic transcyanation reaction of benzaldehyde and acetone cyanohydrin catalyzed by a hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) in an aqueous-organic biphasic system. The optimum pH and

temperature to obtain high *e.e.* were pH 4.0 and 10 °C, respectively. The *Ej*HNL was very stable in ethyl acetate, diethyl ether, methyl-*t*-butyl ether, diisopropyl ether, dibutyl ether, and hexane for 12 h. The best solvent for the highest initial velocity and *e.e.* was diethyl ether with an optimum aqueous phase content of 50% (v/v). Under the optimized conditions, the conversion and *e.e.* of (*R*)-mandelonitrile for 3 h were 40 and 99%, respectively. The aqueous phase containing the enzyme also showed considerably efficient reusability for 4 batch reactions.

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CHAPTER 1

Introduction and Literature Review

Introduction

The demand in chiral intermediate compounds with high enantiomeric purity has been increased drastically in food, chemical, and pharmaceutical industries. In 2000, 35% of intermediates in these industries were chiral and this number is expected to increased to 70% by 2010 (Pollard and Woodley, 2006).

Optically active pure cyanohydrins are important synthetic chiral intermediates in many industrial products such as pharmaceuticals, veterinary products, crop-protecting agents, vitamins and food additives (Hickel *et al.*, 1996). There are various methods for preparation of optically active cyanohydrins. The efficient methods are the asymmetric chemical synthesis employing the chiral catalysts and enzymatic method using hydroxynitrile lyases (HNLs). On a comparison, the enzyme catalyzed synthesis is more efficient in terms of enantiomeric purity (Klempier *et al.*, 1995).

HNLs are important biocatalysts for the enantioselective addition of hydrocyanic acid (HCN) to carbonyl compounds yielding chiral cyanohydrins (Costes *et al.*, 1999). Besides that HNLs can catalyze the decomposition of α-hydroxynitriles or cyanohydrins into HCN and corresponding carbonyl compounds (Wajant and Forster, 1996). These enzymes have been found in several cyanogenic plants such as in plant family Rosaceae, Linaceae, Euphorbiaceae, Olacaceae, Gramineae and Polypodiaceae (Hickel *et al.*, 1996). Furthermore, HNLs which were purified from different plants show different properties. So they are different among their applications on chiral cyanohydrin syntheses (Wajant and Effenberger, 1996), especially, their enantioselectivity and substrate specificity. However, on the synthesis of chiral cyanohydrins the enzyme loses its activity quickly under many reaction conditions such as low pH and high salt concentration in buffer systems. Besides that the enzymes can not be used in aqueous system for

syntheses of hydrophobic products and the high enantiomeric excess can not be obtained. Therefore, two-phase systems have been used to solve these problems (Hickel *et al.*, 1998).

Many families of Thai plants are member of cyanogenic plants (Supavita and Rawdkerd, 1982). So far, there are less research and information on screening and purification of the HNL from Thai plants. Therefore, this research aims to screen, isolate, purify and characterize HNL from cyanogenic plants sources. Furthermore, syntheses of optically active cyanohydrins using the HNL obtained were also studied.

Literature Review

1. Cyanogenesis in plants

Cyanogenesis is the ability of organisms to liberate hydrocyanic acid (HCN). It has been recorded at least in 2,650 higher (vascular) plant species belonging to 550 genera and more than 130 families (Seigler and Brinker, 1993).

All plants produce HCN as a part of their normal metabolism. Small amount of cyanide (less than 1 μM) is produced during the formation of ethylene from 1-aminocyclopropane-1-carboxylic acid, by the action of horseradish peroxidase on L-amino acids and from glyoxylate and hydroxylamine by enzyme catalysis reaction (Kende, 1989 and Poulton, 1990). However, the investigation of cyanogenesis and its distribution in plants deals with higher amount of HCN liberated after breakdown of cyanogenic compounds. Moreover, cyanogenesis has been described in bacteria, cyanobacteria, microalgae and fungi, and also known from arthropod, but no reports of cyanogenesis and cyanogenic glycosides in marine organisms (Scheuer, 1992).

The sources of cyanogenesis in higher plants have been mostly identified as 'cyanogenic glycosides'. In some cases, the cyanogenesis also cause by present of cyanolipids (esters of fatty acids with α- or γ-hydroxynitriles found in seed oils of Sapindaceae plants) and pseudocyanogenic compounds (derivatives of methylazoxymethanol found in plants family Cycadaceae) (Peterson *et al.*, 2000).

1.1. Cyanogenic glycosides

Cyanogenic glycosides are derivatives of α-hydroxynitriles or cyanohydrins (Figure 1). The fairly unstable cyanohydrins are stabilized by β-linked sugar chains. In all cases the first sugar attached to the aglycone is β-D-glucose. The R_1 an R_2 are often different residues resulting in pairs of C-2 epimeric glycosides. Usually, both epimeric forms do not occur in the same plant or even in related species (Nahrstedt, 1993).

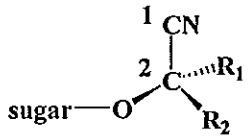


Figure 1. General structure of cyanogenic glycoside.

Source: Lechtenberg and Nahrstedt (1999).

Most reports dealing with cyanogenic glycosides use their trivial names. This is acceptable because of the restricted number of structures (<100) belonging to this group of natural products. The names used are often derived from the latin name of the first reported natural source e.g. amygdalin from *Prunus amygdalus*, prunasin from *Prunus laurocerasus*, triglochinin from *Triglochin maritime* and osmaronin from *Osmaronia cerasiformis*.

A more scientific and systematic nomenclature is the so-called "nitrile-nomenclature" that is also generally accepted. It emphasizes the structural relationships of the cyanogenic glycosides as derivatives of the corresponding nitriles. For example; prunasin is called 2*R*-β-D-glucopyranosyloxy-2-phenylacetonitrile; linamarin is called 2-β-D-glucopyranosyloxy-2-methyl-propionitrile (Lechtenberg and Nahrstedt, 1999).

1.2. Biosynthesis of cyanogenic glycosides

The biosynthesis of cyanogenic glucosides is one of the most investigated metabolic pathways of plant natural products. Biogenetic precursors of most cyanogenic glycosides are protein of five amino acids: L-valine, L-isoleucine, L-leucine, L-phenylalanine, and L-tyrosine (Nahrstedt, 1992).

The general pathway of biosynthesis of cyanogenic glycosides is given by Tapper and Reay (1973) as shown in Figure 2. The α-amino acids are hydroxylated to form 2-oximino acid, or converted to an aldoxime, this in turn to a nitrile. The nitrile is hydroxylated to form an α-hydroxynitrile or cyanohydrin, which is glucosylated to form the corresponding cyanogenic glycoside (McFarlane *et al.*, 1975). 2-Hydroxyisobutyraldoxime, 2-hydroxiphenylacetaldoxime and their glycosides should be considered to be part of a possible alternative pathway of cyanogenic glycoside biosynthesis (Vetter, 2000). All steps in the

biosynthesis of cyanogenic glycosides, except the final glycosylation step, are catalyzed by membrane bound enzyme (Moller and Seigler, 1999).

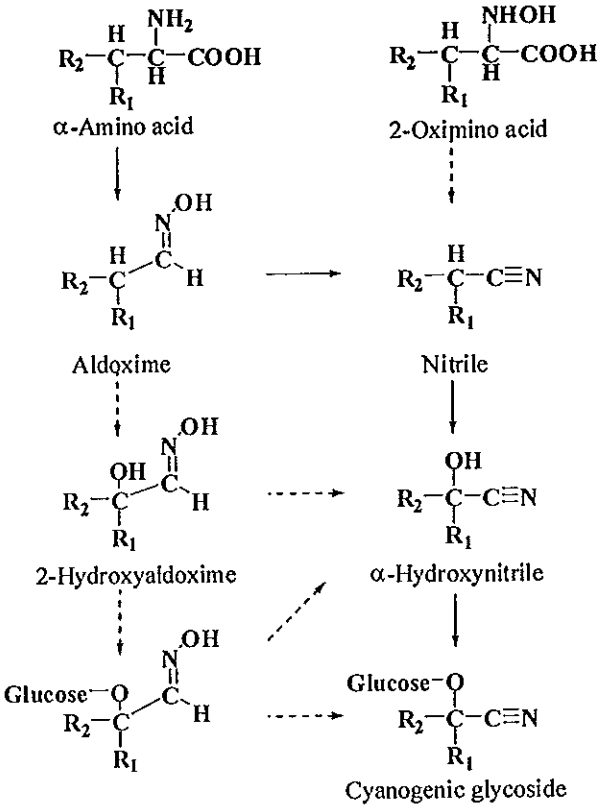


Figure 2. Pathways of cyanogenic glycoside biosynthesis (dotted arrows indicate alternative pathways).

Source: Vetter (2000).

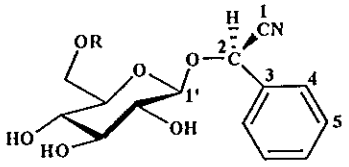
The important changes of biosynthesis of cyanogenic glycosides must take place during the first days of development, these changes is drastically high in monocotyledonous plants. The decrease in HCN production can be demonstrated during the vegetative development of plants when the intensity of photosynthesis increases gradually. The level of HCN in cotyledons of Maceadamia species rose dramatically during germination. The highest concentration was found in the immature first leaf, levels decreased with leaf maturity, correlating with leaf toughening (Vetter, 2000).

1.3. Classification and distribution of cyanogenic glycosides

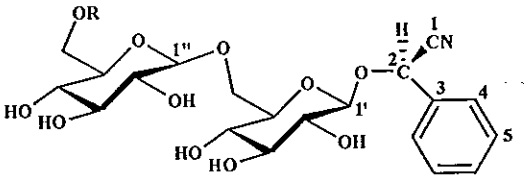
The cyanogenic glycosides are classified according to their putative amino acid, six major groups of cyanogenic glycosides can be derived, the phenylalanine group, the tyrosine group, the valine/isoleucine group, the leucine group, the cyclopenenyl glycine group, and the nicotinic acid group (Lechtenberg and Nahrstedt, 1999).

1.3.1. Cyanogenic glycosides derived from phenylalanine

Cyanogenic glycosides derived from phenylalanine (Figure 3) seem to be widely distributed within the plant kingdom, but there are clear limits of distribution with the Spermatophyta at the subclass level.



R = H	Prunasin	2R
R = H	Sambunigrin	2S
R = Caffeic acid	Grayanin	2R
R = malic acid	Prunasin 6'-malonate	2R



R = H	Amygdalin	2R
R = H	Neoamygdalin	2S
R = p-coumaric acid	Amygdalin 6''-p-coumarate	2R

Figure 3. Cyanogenic glycosides apparently derived from phenylalanine.

Source: Lechtenberg and Nahrstedt (1999).

Phenylalanine derived cyanogens are also rare in monocots. The following plant families contain species with phenylalanine type cyanogenic glycosides: Polypodiaceae, Elaeocarpaceae, Passifloraceae (also cyclopenenyl glycine and valine/isoleucine types), Caricaceae (also cyclopenenyl glycine type), Salicaceae, Sapotaceae, Saxifragaceae, Rosaceae (also leucine and tyrosine types), Mimosaceae (also valine/isoleucine and leucine types),

Fabaceae (also valine/isoleucine and tyrosine types), Myrtaceae, Oliniaceae, Olacaceae, Rutaceae, Convolvulaceae, Lamiaceae, Scrophulariaceae, Rubiaceae, Caprifoliaceae, Aseraceae (also valine/isoleucine type), Liliaceae (Lechtenberg and Nahrstedt, 1999).

The phenyl alanine group is dominated by derivatives of the well known and widespread glycosides prunasin and amygdalin.

1.3.2. Cyanogenic glycosides derived from tyrosine

Cyanogens biogenetically related to L-tyrosine include seven cyanogenic glycosides and two free cyanohydrins (4-glucosyloxy-mandelonitrile and nandinin). Some of these were shown in Figure 4. This group includes the epimeric pair, dhurrin and taxiphyllin, which do not co-occur in the same plant genera (Conn, 1981).

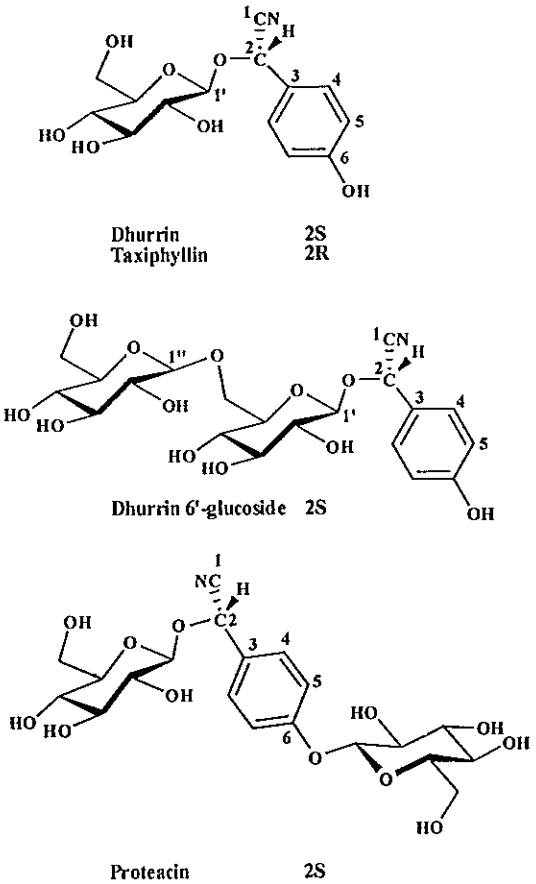


Figure 4. Cyanogenic glycosides apparently derived from tyrosine.

Source: Lechtenberg and Nahrstedt (1999).

The following families contain species with tyrosine type cyanognic glycosides: Cupressaceae, Taxaceae, Magnoliaceae, Calycanthaceae, Ranunculaceae, Berberidaceae, Menispermaceae, Papaveraceae, Platanaceae, Chenopodiaceae, Fabaceae (also valine/isoleucine and phenylalanine types), Proteaceae, Euphorbiaceae (also valine/isoleucine and nicotinic acid types), Boraginaceae, Campanulaceae, and Juncaginaceae (Lechtenberg and Nahrstedt, 1999).

1.3.3. Cyanogenic glycosides derived from valine or isoleucine

A small group of cyanogenic glycosides arises from the aliphatic amino acids L-valine (linamarin and linustatin) and L-isoleucine (lotaustralin, epilotaustralin and neolinustatin). Some members of this type are shown in Figure 5. Linamarin and lotaustralin are widespread and usually co-occur. Diglucoside linustatin was detected in growing seedling, stems and leaves of *Hevea brasiliensis* (Euphorbiaceae) (Selmar *et al.*, 1988).

The following plant families include species with valine/isoleucine type cyanogenic glycosides: Flacourtiaceae, Turneraceae, Passifloraceae, Mimosaceae (also phenylalanine type), Fabaceae (also phenylalanine and tyrosine types), Haloragaceae, Euphorbiaceae (also tyrosine and nicotinic acid types), Linaceae, Asteraceae (also phenylalanine and tyrosine types), Poaceae (also tyrosine and leucine types) (Lechtenberg and Nahrstedt, 1999).

1.3.4. Cyanogenic glycosides derived from leucine

This group of cyanogenic glycoside structures is related to 3-methyl-butyronitrile and contains an asymmetric C-2 carbon (Figure 6). The occurrence of 3-hydroxy-heterodendrin, proacacipetalin, epiproacacipetalin, and proacaciberin is restricted to the genus *Acacia* of Fabaceae (Seigler and Brinker, 1993).

The following plant families contain cyanogenic glycosides derived from leucine: Rosaceae (also phenylalanine and probably tyrosine types), Mimosaceae (also valine/isoleucine and phenylalanine types), Sapindaceae, Poaceae (also tyrosine and valine/isoleucine types) (Lechtenberg and Nahrstedt, 1999).

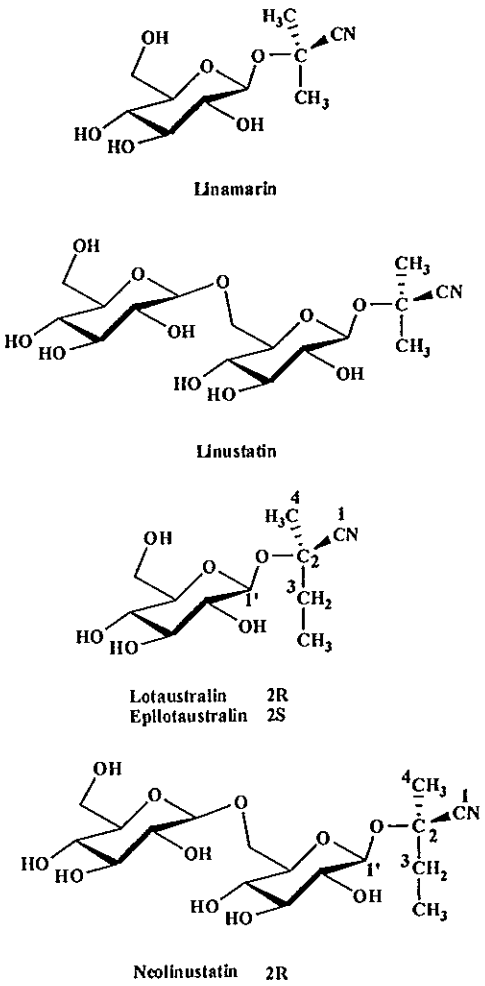


Figure 5. Cyanogenic glycosides apparently derived from valine or isoleucine.

Source: Lechtenberg and Nahrstedt (1999).

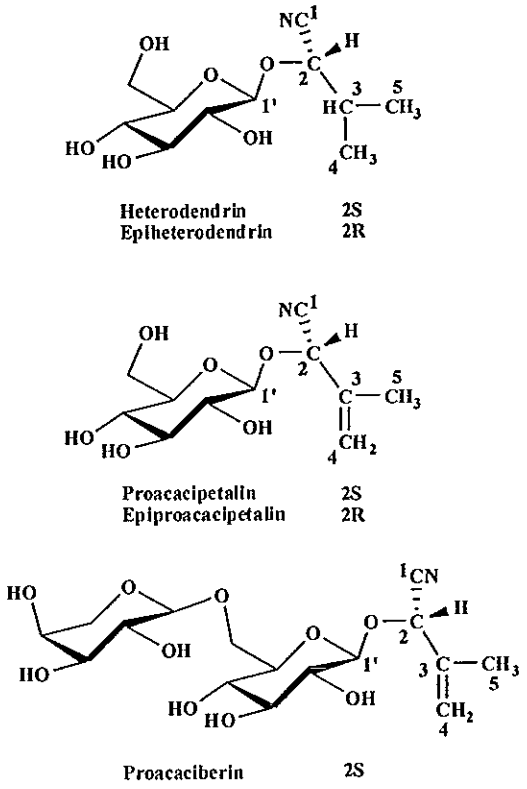
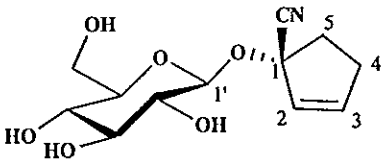


Figure 6. Cyanogenic glycosides apparently derived from leucine.

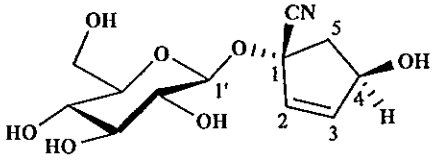
Source: Lechtenberg and Nahrstedt (1999).

1.3.5. Cyanogenic glycosides derived from 2-(2'-cyclopentenyl) glycine

The cyclopentenoid cyanogenic glycosides are derived from the non-protein amino acid 2-(2'-cyclopentenyl) glycine (Figure 7). The occurrence is restricted to a few plant families as follows: Flacourtiaceae (also valine/isoleucine type), Turneraceae (also valine/isoleucine type), Malesherbiaceae, Achariaceae, Passifloraceae (also valine/isoleucine and phenylalanine types), Caricaceae (also phenylalanine type) (Lechtenberg and Nahrstedt, 1999).



Deidaclin 1R
Tetraphyllin A 1S



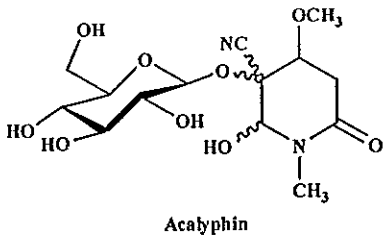
Volkenin (Epitetraphyllin B) 1R, 4R
Tetraphyllin B (Barterin) 1S, 4S
Epivolkenin (Passicoriacin) 1S, 4R
Taraktophyllin (Epipassicoriacin) 1R, 4S

Figure 7. Cyanogenic glycosides apparently derived from 2-(2'-cyclopentenyl) glycine.

Source: Lechtenberg and Nahrstedt (1999).

1.3.6. Cyanogenic glycosides derived from nicotinic acid

In 1982, a new cyanogenic glycoside was isolated from the aerial parts of *Acalypha indica* (Euphorbiaceae, Acalypheae) (Nahrstedt *et al.*, 1982). This compound, acalyphin, represents a new type of cyanogenic glycoside apparently derived from nicotinic acid (Figure 8). Acalyphin is a cyanopyridone derivative. The biogenetic precursors indicating that compounds of this type are biochemically connected to the nicotinamide adenine dinucleotide (NAD) cycle (Lechtenberg and Nahrstedt, 1999).



Acalyphin

Figure 8. Cyanogenic glycoside apparently derived from nicotinic acid.

Source: Lechtenberg and Nahrstedt (1999).

1.4. Cyanogenesis by catabolism of cyanogenic glycosides

The term of "cyanogenesis" means not only the synthesis of cyanogenic glycosides, but including the enzymatic hydrolysis of cyanogenic glycoside into free HCN and corresponding compounds (Vetter, 2000).

The cyanogenic glycosides are hydrolyzed chemically in acidic solutions and enzymatically by β-glucosidases. The products obtained after hydrolysis are the sugar and the corresponding cyanohydrin (α-hydroxynitrile) that itself is unstable and prone to decompose non-enzymatically in the alkaline condition (Poulton, 1990; Fomunyan *et al.*, 1985). The dissociation of cyanohydrins also occurs enzymatically catalyzed by a 'hydroxynitrile lyase', to give a carbonyl compound and HCN (Vetter, 2000).

In plants, cyanogenesis in intact plants is prevented by compartmentation of cyanogenic glycosids and catabolic enzymes at subcellular or cellular level, therefore no HCN is released. Until the tissue is disrupted, the leaked enzyme can catalyze the substrate, and then cyanogenesis occurs. In *Sorghum* leaves, cyanogenesis is prevented by compartmentation at the tissue level. The β-glucosidase (dhurrinase) and the hydroxynitrile lyase are located in the cytoplasm of mesophyll cells whereas the substrate dhurrin is in the epidermal cells, especially in vacuoles (Saunders *et al.*, 1977; Kojima *et al.*, 1979; Thayer and Conn, 1981). There are other examples of subcellular compartmentation where the substrate is concentrated in vacuole of the cell and the β-glucosidases are cell wall-bound enzyme as observed for *Trifolium repens* (Kakes, 1985), the Costa Rica lima bean (Frehner and Conn, 1987) or *Hevea brasiliensis* (Selmar *et al.*, 1989). In *Prunus serotina*, amygdalin hydrolase was found in the procambial cell, prunasin hydrolase in the peripheral layer meristem tissues and hydroxynitrile lyase in the protein bodies of the cotyledonary parenchyma cells (Swain *et al.*, 1992; Poulton 1993). The substrate amygdalin is localized in cotyledonary parenchyma cells (Poulton and Li, 1994). Disruption of the tissue or wounding of the cells is necessary to initiate the cyanogenesis.

Catabolism of cyanogenic glycosides plays an important role on plant defence against herbivores and microbial attack, or as a nitrogen source for amino acid synthesis (Hickel *et al.*, 1996). If the subcellular structures are destroyed, the catabolic enzymes can act on the cyanogenic glycosides. As shown in Figure 9, the catabolism is initiated by cleavage of the

carbohydrate moiety by β-glucosidase. This cleavage can be simultaneous or sequential depending on the type of plant and tissue. After the cleavage of carbohydrate moiety, the cyanohydrins intermediates may decompose either spontaneously or enzymatically, in the presence of a 'hydroxynitrile lyase', yielding HCN and corresponding ketones and aldehydes (Moller and Seigler, 1999). However, the presence of hydroxynitrile lyases can increase the dissociation velocity of cyanonydrins 10-20 folds in acidic media. Furthermore, it is also note that the protecting properties of cyanogenic glycosides are not only due to HCN, which is an effective inhibitor of respiration and of enzymes containing metals, but also the inhibition of liberated ketones and aldehydes on enzymes can often possess cytotoxic activities (Hickel *et al.*, 1996)

Cyanogenic glycosides may also be catabolized without release of HCN. For example, during the germination of *Hevea brasiliensis* the total amount of cyanogenic compounds in seeds decreases greatly but HCN is not liberated from the seeds. The decrease of cyanogenic glycosides is not due to cyanogenesis, but it is caused by the conversion of cyanogenic glycosides to non-cyanogenic compounds (Figure 9). HCN is refixed by β-cyanoalanine synthetase (EC 4.4.1.9) and forms β-cyanoalanine with L-cysteine. The β-Cyanoalanine is then hydrolysed by β-cyanoalanine hydrolase to L-asparagine. Consequently, HCN can be considered as nitrogen source for the amino acid synthesis of young seedling tissues (Lieberei *et al.*, 1985, Selmar, 1993).

In mammalian cells, microorganisms, insects, a further reaction for the refixation or detoxification of HCN is known. The enzyme rhodanese (thiosulfate cyanide sulfurtransferase, EC 2.8.11) catalyses the thiosulfate and cyanide into the less toxic compounds thiocyanate (rhodanid) and sulfite (Okolie and Obasi, 1993).

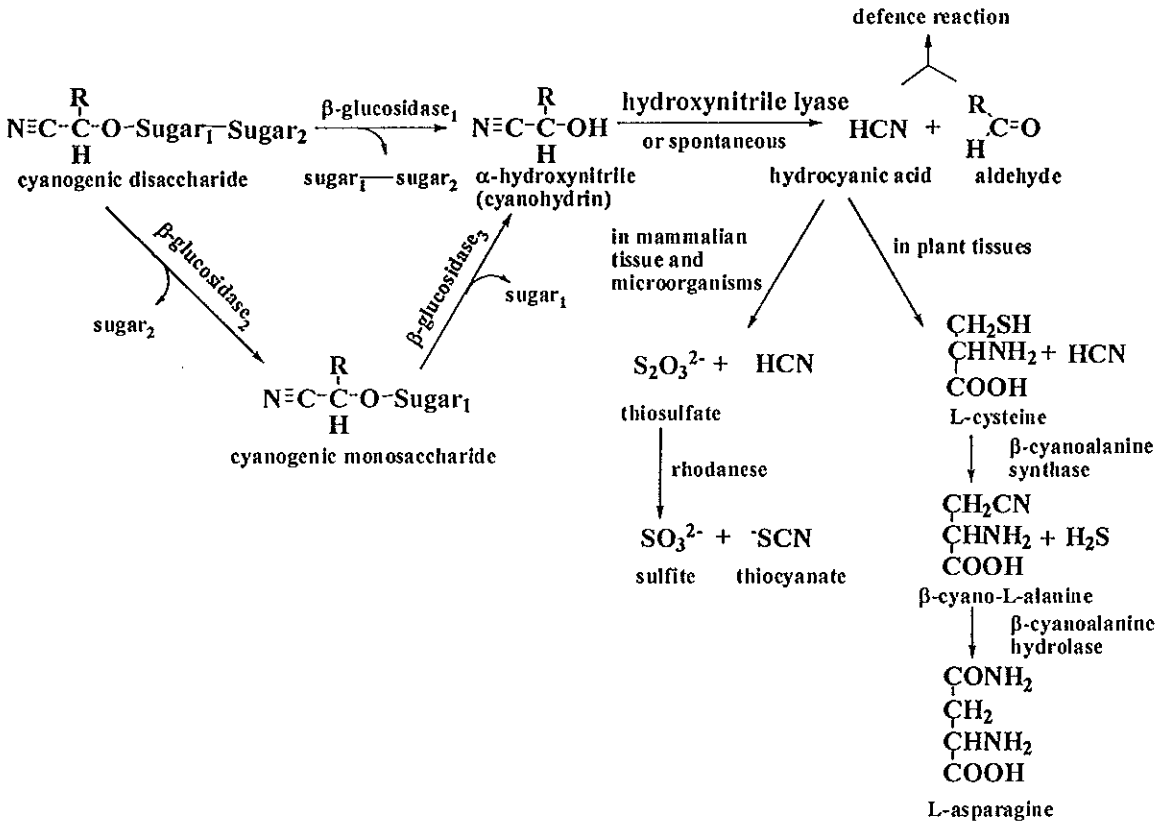


Figure 9. Catabolic pathway of cyanogenic glycosides.

Source: Hickel *et al.* (1996).

1.5. Determination of cyanide releasing from cyanogenic plants

Many methods have been developed for determination of the total cyanide content of cyanogenic plants such as titration of cyanide solution after extraction from plant materials with acid or alkaline silver nitrate (AOAC, 1965), trapping the cyanide gas in alkaline solution and measure the cyanide content by cyanide-sensing electrode (Gillingham *et al.*, 1969), employing HPLC consisting of anion exchange column and electrochemical detector (Chadha *et al.*, 1995; Kobaisy *et al.*, 1996), colorimetric assay of isonicotinate-1,3-dimethylbarbiturate or piridin/pyralozone color reagents (Essers *et al.*, 1993), Feigl-Anger spot test which may be inhibited by tannin (Van Wyck, 1989; Goldstein and Spencer, 1985), and acid hydrolysis of cyanogenic glycosides (Bradbury *et al.*, 1994). These methods are employed the expensive

equipments, required many steps, use several toxic compounds, and difficult to use in survey for cyanogenesis in a wide range of plants.

The classical picrate method or Guignard method is based on the reaction of alkaline picrate paper with HCN liberated in a closed test tube by spontaneous enzymatic hydrolysis of crushed plant materials (Guignard, 1906). Cyanide can be detected at 30-50 μg/g concentration. The picrate method was found to be more accurate and reproducible and easily to use in the field survey (Haque and Bradbury, 2002).

Picrate method is employed the reaction of cyanide and picric acid (2,4,6-trinitrophenol) yielding the brown color of isopurpuric acid (2,6-dinitro-5-hydroxy-4-hydroxylamino-1,3-dicyanobenzene) (Williams and Edwards, 1980) as shown in Figure 10.

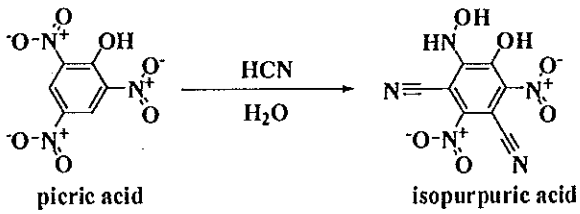


Figure 10. Reaction of picric acid and hydrocyanic acid yielding isopurpuric acid.
Source : William and Edwards (1980).

2. Hydroxynitrile lyases

Hydroxynitrile lyases (HNLs) have attracted the attention of bioorganic scientists since the industrial application of these enzymes as biocatalysts for the synthesis of enantiomerically pure cyanohydrins (α-hydroxynitrile) (Hickel *et al.*, 1996).

HNLs are involved in catabolism of cyanogenic glycosides in cyanogenesis. Their natural function is the catalysis of cyanohydrin cleavaged from cyanogenic glycosides into HCN and the corresponding aldehydes or ketones (Krammer *et al.*, 2007). As the reversible reaction of HNLs, the enzyme can also be used for the synthesis of enantiomerically pure cyanohydrins. These cyanohydrins are valuable structures for the chemical synthesis of pharmaceutical intermediates such as ACE-inhibitors, antibiotics, ephedrine, biotin, pantothenic acid, etc (Kruse, 1992).

2.1. Classification of hydroxynitrile lyases

HNLs can be classified according to the different properties of the enzyme such as (*R*)- or (*S*)-specificity and FAD (flavine-adenine dinucleotide) or non-FAD-containing enzymes. In this review, HNLs are classified according to the EC number based on the chemical reaction of the enzyme.

2.1.1. Mandelonitrile lyase (EC 4.1.2.10)

Mandelonitrile lyase is a (*R*)-HNL which catalyzes the cleavage of (*R*)-mandelonitrile to benzaldehyde and hydrocyanic acid. Mandelonitrile lyases were mainly isolated from the Rosaceae subfamilies Prunoideae and Maloideae (Hickel *et al.*, 1996) such as *Prunus amygdalus*, *Prunus serotina*, *Prunus mume*, *Chaenomles sinensis*, etc (Asano *et al.*, 2005; Worker *et al.*, 1994; Hu and Poulton, 1999). The natural substrates of this enzyme are (*R*)-(+)-mandelonitrile derived from cyanogenic glycosides (*R*)-prunasis or amygdalin (Conn, 1981). Moreover, the other plants containing this enzyme were also reported such as *Cucumis melo*, *Cydonia oblonga*, and *Passiflora edulis* (Asano *et al.*, 2005; Hernández *et al.*, 2004).

2.1.2. *p*-Hydroxymandelonitrile lyase (EC 4.1.2.11)

p-Hydroxymandelonitrile lyase is a (*S*)-HNL catalyzing the decomposition of (*S*)-mandelonitrile and (*S*)-4-hydroxymandelonitrile derived from the cyanogenic glycoside (*S*)-sambunigrin and (*S*)-dhurrin, respectively, in cyanogenic plants. This enzyme was found in the plant extracts of *Ximemia americana*, *Sorghum bicolor*, *Annona cherimola*, *Annona squamosa*, and *Baliospermum montanum*. Only HNLs from *X. americana* and *S. bicolor* were purified and characterized (Asano *et al.*, 2005; Hernández *et al.*, 2004; Wajant *et al.*, 1995; Kuroki and Conn, 1989).

2.1.3. Acetone cyanohydrin lyase (EC 4.1.2.37)

Acetone cyanohydrin lyase is a (*R*)-HNL catalyzing the degradation of acetone cyanohydrin in *Linum usitatissimum* (Albrecht *et al.*, 1993).

2.1.4. Hydroxynitrilase (EC 4.1.2.39)

Hydroxynitrilase is (*S*)-HNL catalyzing the decomposition of acetone cyanohydrin and 2-butanone cyanohydrin. This enzyme was found in *Hevea brasiliensis* and *Manihot esculenta* (Wajant and Förster, 1996; Hughes *et al.*, 1994).

2.2. Catalytic reaction of hydroxynitrile lyases

2.2.1. Decomposition of cyanohydrins

In the cyanogenic plants, HNLs catalyzes the decomposition of cyanohydrins yielding the rapid releasing of HCN in the injured cyanogenic plants. HNLs are stereoselectivity enzymes, therefore the (*R*)- and (*S*)-HNLs catalyzed specifically on the cleavage of (*R*)- and (*S*)-cyanohydrins, respectively. The cyanohydrins decomposition catalyzed by HNLs is shown in Figure 11.

2.2.2. Synthesis of cyanohydrins

HNLs do not only catalyze the cleavage of cyanohydrins, but also the enantioselective addition of HCN to aldehydes or ketones yielding optically active cyanohydrins (Effenberger, 1998). (*R*)-HNL catalyzes the addition of HCN to carbonyl compound, aldehydes or ketones yielding the (*R*)-cyanohydrins, while (*S*)-HNL catalyzes the formation of (*S*)-cyanohydrins (North, 2003) as shown in Figure 12.

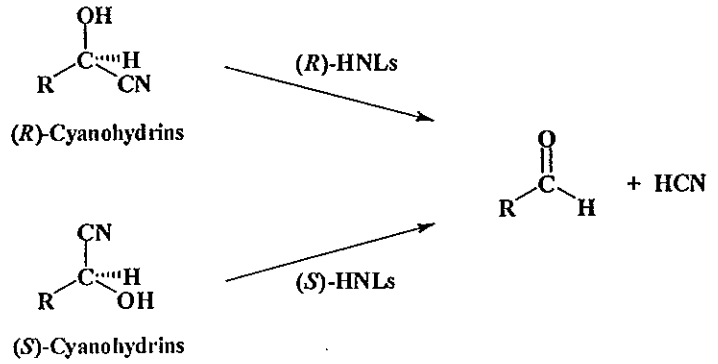


Figure 11. Hydroxynitrile lyases catalyze the enantioselective decomposition of cyanohydrins.
Source: North (2003).

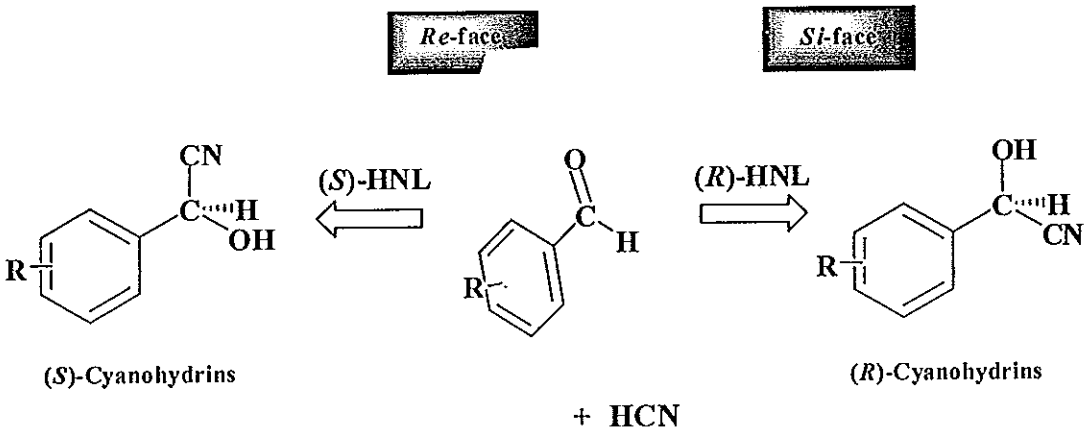


Figure 12. Hydroxynitrile lyase catalyzes enantioselective cyanohydrin synthesis.
Source: Kruse (1992).

2.3. Reaction mechanism of hydroxynitrile lyases

Catalytic reactions of HNLs from *Hevea brasiliensis* (*Hb*HNL) and *Manihot esculenta* (*Me*HNL) are very similar and proceed by nucleophile displacement. The *Hb*HNL uses the catalytic triad of Ser-80, His-235 and Asp-207 residues, while catalytic triad of *Me*HNL consists of Ser-80, His-236, and Asp-208. In both cases, His acts as an active species and increases the basicity of Ser resulting in the abstraction of a proton from the hydroxyl group of

cyanohydrin (Figure 13). In the case of *Hb*HNL, the proposed catalytic mechanism showed that Lys-236 plays a critical role in catalytic action at low pH values by stabilizing the negative charge of cyanide ion, which is present in direct contact with cyanide moiety of the substrate (Gruber, 2001; Lauble *et al.*, 2002).

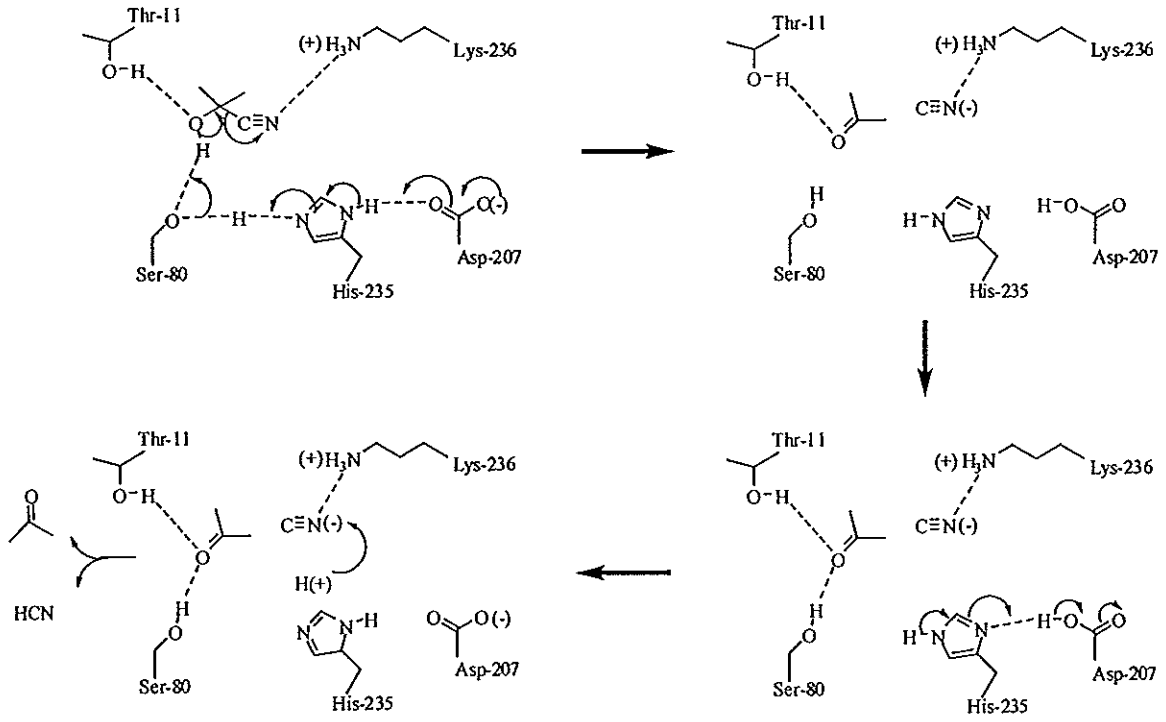


Figure 13. Reaction mechanism of hydroxynitrile lyase from *Hevea brasiliensis*.
Source: Gruber (2001).

For the catalytic reaction of FAD-HNLs, the reaction mechanism of HNL from *Prunus amygdalus* (*Pa*HNL) was proposed as shown in Figure 14 by Dreveny *et al.* (2002). This reaction relies on general acid/base catalysis by imidazole of a conserved catalytic His-497 which acts as a base and deprotonates the hydroxyl group of the substrate. Negativity of cyanide ion is neutralized by pronounced positive electrostatic potential at the active site by two positively charged residues Arg-300 and Lys-361. The *Pa*HNL binds FAD cofactor in its oxidized form. The reversal of electrostatic potential upon FAD removal or modification makes the complex unstable at active site leading to inactivation of the enzyme. Moreover, *Pa*HNL follows uni-bi mechanism in which aldehydes binds first to active site followed by HCN.

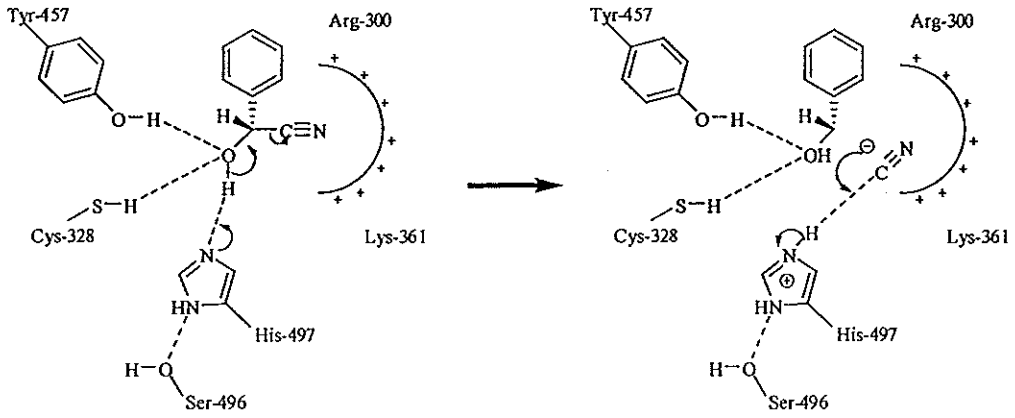


Figure 14. Reaction mechanism of hydroxynitrile lyase from *Prunus amygdalus*.

Source: Dreveny *et al.* (2002).

The catalysis in HNL from *Sorghum bicolor* (*Sb*HNL) was reported by Laubel *et al.* (2002). Hydrogen bonds are formed between substrate hydroxyl group, Ser-158 and oxygen atom of Trp-270-carboxyl group. The carboxylate moiety of tryptophan acts as catalytic base causing abstraction of proton from cyanohydrin hydroxyl group, while water at active site transfers this proton to cyanide resulting in carbon-carbon cleavage (Figure 15).

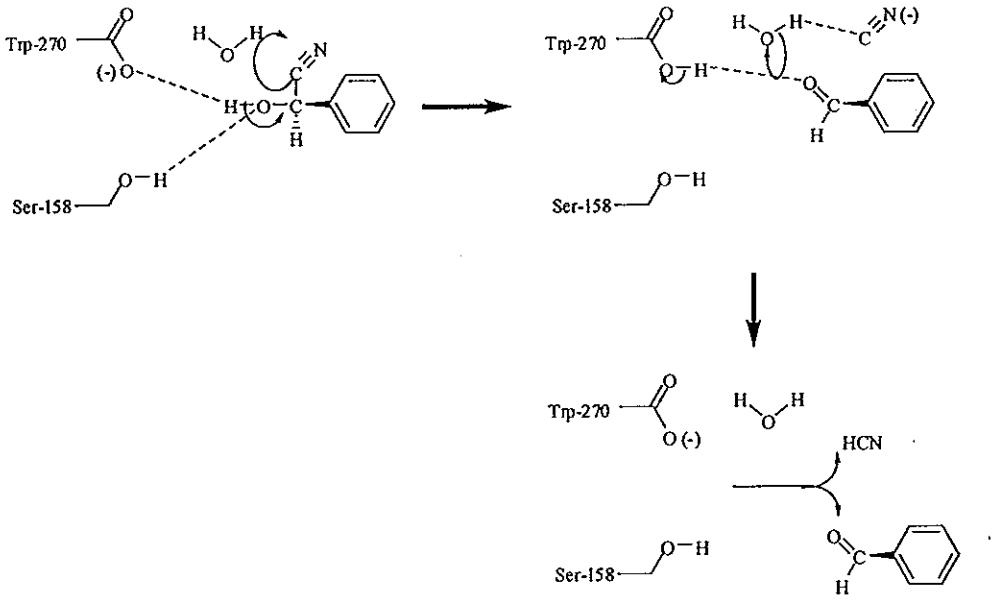


Figure 15. Reaction mechanism of hydroxynitrile lyase from *Sorghum bicolor*.

Source : Lauble *et al.* (2002).

2.4. Characteristics of hydroxynitrile lyase

HNLs have been found in several cyanogenic plants family such as Rosaceae, Linaceae, Euphorbiaceae, Olacaceae, Gramineae and Polypodiaceae (Hickel *et al.*, 1996). HNLs purified from different plants showed the difference in their characteristics. The different characteristics of reported HNLs were described and summarized in Table 1.

2.4.1. Prosthetic group and glycosilation

HNLs have also been characterized into two groups, one which contains flavin-adenine dinucleotide (FAD) as a prosthetic group and the other one lack of it.

The flavoprotein HNLs are found only in members of the plant family Rosaceae, e.g. cherry, peach, apricot, almond, plum, and apple (Hickel *et al.*, 1996). These enzymes are single-chain glycoprotein containing up to 30% carbohydrate *N*-glycosidically bound to the protein. The active site of the enzyme contains the non-covalently bound FAD as a prosthetic group (Gerstner and Pfeil, 1972). FAD in an oxidize state appears to be an essential component for maintenance the overall conformation structure and bound near catalytic site, but does not involve in a redox reaction (Becker *et al.*, 1963, Jorns, 1979).

The FAD-independent HNLs are more heterogeneous in structure and have been isolated from different plant families. They differ in carbohydrate content in the enzyme molecule. HNLs isolated from *Ximenia americana* and *Sorghum bicolor* are glycoprotein. *Sb*HNL contains 7% of *N*-linked carbohydrate (Hickel *et al.*, 1996).

2.4.2. Molecular mass, subunit and isozyme

(*R*)-HNLs have been purified from varieties of *Prunus* sp. (Rosaceae). The native molecular mass of these enzymes is ranging from 50-80 kDa. The (*R*)-HNLs consist of a single subunit. However, isozymes of (*R*)-HNLs were reported and showed slightly different in some characteristics such as p*I* or glycosidic side chain (Xu *et al.*, 1986; Yemm *et al.*, 1986; Jansen *et al.*, 1992; Hickel *et al.*, 1996; Wajant and Effenberger, 1996).

The (*R*)-HNL purified from flax, *Linum usitatissimum*, (*Lu*HNL) has molecular mass as 82-87 kDa by gel fitration and 42-43 kDa by SDS-PAGE and exist in a homodimeric conformation (Xu *et al.*, 1988; Albrecht *et al.,* 1993; Hickel *et al.*, 1996; Wajant and Effenberger, 1996).

The purified (*R*)-mandelonitrile lyase from the fern *Phlebodium aureum* is a homomultimer with subunits molecular mass of 20 kDa in SDS-PAGE under reducing and non-reducing condition and the apparent molecular mass of the native isoenzymes estimated by gel filtration on Superdex 200 was 168 ±30 to 40 kDa (Wajant *et al.*, 1995).

In *Hevea brasiliensis*, the purified *Hb*HNL consists of non-covalently linked monomers of 30 kDa under reducing and non-reducing conditions of SDS-PAGE with a native molecular mass between 100-105 kDa determined by gel filtration indicated that *Hb*HNL is a homotrimer or homotetramer (Wajant and Forster, 1996; Wajant and Effenberger, 1996). Nevertheless, with respect to molecular mass, subunit composition and glycosylation, *Hb*HNL is different from most other non-flavoprotein HNLs, which are heterotetrameric (*Sb*HNL), homodimeric (*Lu*HNL) or monomeric holoenzymes. However, there is considerably more biochemical similarity between *Hb*HNL and *Me*HNL. Both enzymes are homomers of non-glycosylated 30 kDa subunits (Wajant and Forster, 1996).

Hickel *et al.* (1996) reported, based on the experiment of Hughes *et al.* (1994), the purified HNL from *Manihot esculenta* Crantz (*Me*HNL) is homotrimer or homotetramer with native molecular mass ranging from 92-124 kDa and subunit molecular mass by SDS-PAGE is between 28.5-30 kDa. Chueskul and Chulavatnatol (1996) studied on HNL form petioles of cassava. The purified *Me*HNL is a homotetramer with a subunit molecular mass of 25.6 kDa by SDS-PAGE and the native molecular mass of 102 kDa by Sephadex G-200 column. The purified *Me*HNL (White *et al.*, 1998) had a subunit molecular mass of 28.5 kDa, similar to that reported by Hughes *et al.* (1994). The native enzyme, however, had a molecular mass of 50.1 kDa, suggesting that it was a dimer. These results are in contrast to those of Hughes *et al.* (1994) and Chueskul and Chulavatnatol (1996). The difference in native molecular mass may be attributed to difference in the ionic strength of the buffer used for size-exclusion chromatography. Hughes *et al.* (1994) reported that higher-ionic-strength buffers facilitate the formation of higher-ordered

homomeric complexes. Chueskul and Chulavatnatol (1996) observed a homotetrameric native enzyme structure by gel filtration in the absence of salts.

On the purified (*S*)-hydroxynitrile lyase from *Sorghum bicolor* L. (*Sb*HNL), the molecular mass of the native enzyme was determined as 95-105 kDa. On SDS-PAGE showed molecular mass of non-covalent bond subunits is 33±1 kDa, and 55±2 kDa for non-reduced subunits indicated that it is heterotetramer (Jansen *et al.*, 1992; Wajant and Mundry, 1993; Hickel *et al.*, 1996).

Mandelonitrile lyase from *Ximenia americana* L. (*Xa*HNL) was purified and studied. Native and subunit molecular weights of 38 and 36.5 kDa, respectively, suggest the enzyme is a monomer (Kuroki and Conn, 1989).

2.4.3. Optimum pH and pH stability

The pH optimum of HNL from *Prunus* sp. is ranging from 5.0-7.0, and p*I* is in the range of 4.2-4.85 (Hickel *et al.*, 1996). The HNLs from *Prunus serotina* Ehrh. (*Ps*HNL) and *Prunus lyonii* (*Pl*HNL) showed pH optima at 6.0-7.0 and 5.5-6.0, respectively (Yemm and Poulton, 1986). Jansen *et al.* (1992) observed that the enzyme exhibit stability over a wide pH range from pH 3-11.

*Sb*HNL exhibits stability over a wide pH range from pH 2-11 (Jansen *et al.*, 1992) and pI is 4.0 (Hickel *et al.*, 1996).

*Lu*HNL has the pH optimum at approximately 5.5 (Xu *et al.*, 1988) and the enzyme was stable over the pH range 6-11 during storage. But in the acidic pH range the enzyme is much less stable than the HNL form almond. The half-life of the native enzyme was measured approximate 1 h at 25 °C in 50 mM citrate buffer, the HNL was most stable at pH 5.0 and decreased activity rapidly in pH 4.0 and 3.0 (Albrecht *et al*, 1993). The isoelectric point of the enzyme was in the range of pH 4.5-4.85 according to chromatofocusing (Xu *et al.*, 1988; Albrecht *et al.*, 1993; Hickel *et al.*, 1996).

pH optimum, pH stability of Hnl from the *Phlebodium aureum* was determined only below pH 7.5 because of the base-catalysis decomposition of mandelonitrile. The activity

generally increased from pH 3.5 to 6.2 and leveled off between pH 6.2 and 7.0 (Wajant *et al.*, 1995).

Wajant and Forster (1996) reported that the pH optimum of *Hb*HNL using the natural substrate acetone cyanohydrin was between 5.3 and 5.7. Therefore, *Hb*HNL can effectively accelerate the dissociation of acetone cyanohydrin in damaged tissue of *Hevea brasiliensis* where the pH is slightly acidic. The physiological importance of rapid cyanogenesis is shown by the fact that only hevea plants possessing high HNL concentration are able to release HCN efficiently. Therefore, repellent action of cyanogenic hevea plants depends on both the final level of HCN as well as the rate of acetone cyanohydrin cleavage.

In the studies of cassava HNL, the optimum pH is 5.0-5.4. Chueskul and Chulavatnatol (1996) reported that HNL from cassava petiole showed a sharp pH profile with an optimum at pH 5.0. The activity of cassava HNL was quite stable at room temperature for 2 hours in the pH range of 6-11. In the pH range of 6 to 4, the HNL activity decreased gradually. At pH 4, the activity was 10% that at pH 6 (Chueskul and Chulavatnatol, 1996). The HNL from cassava has 3 isoforms and p*I* value of the enzymes are between 4.1-4.6 (Hickel *et al.*, 1996). Hughes *et al.* (1994) determined that cassava HNL has three isoforms, a major isoform with p*I* 4.4 and two minor isoforms with p*I* 4.1 and 4.6 as same as the report of Chueskul and Chulavatnatol (1996). Since the material for isolation of the enzyme was collected from a number of different plants, the existence of isoforms could be due to allerlic variation between plants. Alternatively, there may be more than one isoform produced by each plant (Hughes *et al.*, 1994).

Kuroki and Conn (1989) found that the HNL from *Ximenia americana* L. had a pH optimum of 5.5 and the isoelectric point was pH 3.9 as determined by isoelectric focusing. This report is similar to Hickel *et al.* (1996), the p*I* of *Ximenia* HNL is between 3.9-4.0.

2.4.4. Optimum temperature and temperature stability

On the studies of enzyme stability at 4 °C, *Prunus lyonii* HNL was stable for at least 180 days and *Linum usitatissimum* HNL was stable at least 45 days without any appreciable loss of enzyme activity (Xu *et al.*, 1986; Xu *et al.*, 1988).

The temperature stability of *Phlebodium aureum* HNL was found to be active over a broad range of temperature with maximal activity at 35 to 40 °C (Wajant *et al.*, 1995).

At pH 6.5, the HNL from *Hevea brasiliensis* was stable for hours at 30 °C whereas above 70 °C the enzyme is inactivated rapidly (Bauer *et al.*, 1998).

Sorghum bicolor HNL showed a good resistance against heat inactivation is observed. The remaining activity after 60 min at 60 °C for the enzyme was higher than 60%, complete inactivation was observed at 70 °C after 30 min (Jansen *et al.*, 1992).

Ximenia americana HNL was stable at 4 °C and at room temperature for at least 1 month (Kuroki and Conn, 1989).

2.4.5. Effect of additives

Yemm and Poulton (1986) observed the rate of enzymatic mandelonitrile degradation in almond HNL, it was unaffected by $MgCl_2$, $MnCl_2$, $ZnCl_2$, Na_2EDTA at 10 mM concentration. The enzyme was inhibited by $FeSO_4$ (10 mM) or $PbNO_3$ (0.1 mM), but slight and comparable inhibitions (5-10%) were elicited by $AgNO_3$ and $CuSO_4$ (0.1 mM). The almond HNL was sensitive to $HgCl_2$, showing 91% inhibition at 0.01 mM. These data suggested that metal ions are not required for enzyme activity. It was strengthened by the observation that the enzyme was sensitive to 2,2'-dipyridyl (0.1 mM) or o-phenanthrolin (10 mM). However, it was inhibited to similar extents by β-mercaptoethanol (17% at 10 mM) and iodoactic acid (42% at 1 mM) but was unaffected by iodoacetamide (1mM) and ρ-chloromercuribenzoate (1 mM). Participation of a cysteine residue in the reaction was proposed. Besides that sodium cyanide and the cyanogens amygdalin and prunasin (1 mM) failed to inhibit lyase activity. Benzoic acid, *p*-hydroxybenzyl alcohol, and benzyl alcohol (50 mM) were the most potent inhibitors (40-60% inhibition) of HNL. Benzoate and benzyl alcohol were previously shown to be competitive inhibitors of HNL from sweet almond lyase. Aromatic metabolites bearing hydroxyl groups at either the *para* or *ortho* positions inhibited lyase at 50 μM, but to lesser extents than the unsubstituted parent compounds. Aromatic compounds having a *meta*-hydroxy substitution pattern were ineffective lyase inhibitors. As with sweet almond lyase, mandelic acid and hexanoic acid were slightly inhibitory while mandelamide was inactive.

The effects of several compounds on *Linum usitatissimum* HNL were examined. At the concentrations tested, the sulfhydryl reagents 2-mercaptoethanol (10 mM), iodoacetamide (1 mM), and iodoacetic acid (1 mM) exhibited almost no inhibition of the lyase activity. Isobutyronitrile (10 mM) and isopropyl alcohol (10 mM) also failed to have any effect on the lyase activity (Xu *et al.*, 1988).

The influences of various additives on the enzymatic activity of *Phlebodium aureum* HNL were determined. This enzyme was not affected by $AgNO_3$, and iodoacetamide treatment resulted in only modest inhibition. Likewise, the Ser-modifying agents, diisopropylfluorophosphate (DFP) and phenylmethylsulphonyl fluoride (PMSF) showed a less effect on enzymatic activity indicating that no Cys or Ser involved in catalysis, whereas it was only poorly or not at all affected by DTT, EDTA, EGTA, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CuSO_4$. But for diethyl pyrocarbonate (DEP), a reagent capable of reacting with the imidazole group of His, indicating the involvement of this amino acid in catalysis (Wajant *et al.*, 1995).

The stability of HNL from rubber tree could be improved with all four tested additives (sorbitol, sucrose, lactose and glycerine) with increasing concentration (50-400 mg/ml). Sorbitol improves stability even six times but only at high concentrations (400 mg/ml). With all other additives, a maximal three fold increase in half-life could be achieved. Improved enzyme stabilization by sugars and polyols has been frequently reported assuming that the addition of these additives strengthens the hydrophobic interactions among non polar amino acid residues making them more resistant against unfolding (Bauer *et al.*, 1998). Additives such as the natural *H. brasiliensis* plant extract as well as fern extract stabilized the enzyme very effectively even at very low concentrations. No loss of activity was determinable at pH 3.75 during 1 h. Saccharose and sorbit also stabilized the HNL but not so effectively as the plant matrices. (Hickel *et al.*, 1997). Inhibitor studied using various serine/cysteine and histidine modifying reagents suggest the involvement of these residues also in other HNLs, on the studied of Wajant and Forster (1996), examined the effect of DFP (active site serine reagent) and DEP (active site histidine reagent), on the activity of *Hevea brasiliensis* HNL. Both reagents were found to inhibit the enzyme activity, indicating the involvement of these residues in the active site of the HNL.

HNL from cassava petiole was studied. Several alcohols, aldehydes, and ketones were found to inhibit the HNL activity. However, different effects on the kinetic parameters of

the HNLs were noted among the inhibitors. The alcohols caused large increases in the K_m value for acetone cyanohydrin but little changes in th V_{max} value. On the other hand, the aldehydes strongly suppressed the V_{max} value with slight effects on the K_m value. Like the alcohols, the ketone inhibitors caused large increases in the K_m but not the V_{max} values. Based on these data, the alcohols and the ketones were competitive inhibitors of the HNL, whereas the aldehydes were noncompetitive inhibitors. Furthermore, the estimated K_i values of these inhibitors showed that the inhibitors containing 4 carbon atoms exhibited a stronger effect (a lower K_i) than those containing fewer or more than 4 carbon atoms but having the same functional group. In addition, among those containing 4 carbon atoms, the aldehyde inhibitor was more potent (lower K_i) than the corresponding alcohol inhibitor which in turn was stronger than the ketone inhibitor (Chueskul and Chulavatnatol, 1996).

Effect of additives on *Ximenia* HNL was assayed, cation appear not to be required for lyase activity, indicated by the observation that lyase was not effected by metal chelators, EDTA (5 mM), 2-phenanthroline (0.1 mM), and α,α'-dipyridyl (0.1 mM). Assays performed in the presence of a variety of metal salts ($ZnCl_2$, $MnCl_2$, $MgCl_2$, $CuSO_4$, $HgCl_2$, $AgNO_3$, $FeCl_2$, $FeCl_3$ and $Pb(NO_3)_2$ at 0.1 mM) supported the lack of a metal-ion requirement. However, the *Ximenia* HNL was inhibited by $AgNO_3$ (50% inhibition at 0.1 mM). This enzyme also exhibited 27% inhibition by the reduced glutathione (10 mM) and the *p*-chloromecuriphenylsulfonic acid (1 mM) (Kuroki and Conn, 1989).

Table 1. Properties of purified hydroxynitrile lyases.

Plant source	Stereoselectivity and abbreviation	Molecular mass (kDa) (Subunits)	Isozyme	pI	pH optimum	Co-enzyme	*N*-glycosylation
Prunus amygdalus (Rosaceae)	(*R*)-*Pa*HNL	Native : 72±2 SDS : 72±2 (single)	4	4.3-4.5	5.0 – 6.0	FAD	Mannose
Prunus laurocerasus (Rosaceae)	(*R*)-*Pl*HNL	Native : 60 SDS : (-)	3	4.2-4.4	5.5-6.0	FAD	Glucose and galactose
Prunus lyonii (Rosaceae)	(*R*)-HNL	Native : 50 SDS : 59	(-)	4.75	5.5	FAD	(-)
Prunus serotina (Rosaceae)	(*R*)-*Ps*HNL	Native : 55.6 SDS : 57-59 (monomeric)	5	4.58-4.63	6.0-7.0	FAD	Glucose and/or mannose
Linum usitatissimum (Linaceae)	(*R*)-*Lu*HNL	Native : 82-87 SDS : 42-43 (homodimer)	Yes	4.5-4.85	5.5	No	No
Phlebodium aureum (Filitaceae)	(*R*)-*Pha*HNL	Native: 168±30 SDS : 20 (multimer)	3	(-)	6.5	No	(-)
Sorghum bicolor (Poaceae)	(*S*)-*Sb*HNL	Native: 108±3 SDS : 55±2 (heterotetramer)	3	4.0	(-)	No	(-)
Sorghum valgare (Poaceae)	(*S*)-HNL	Native: 180	(-)	(-)	5.0-6.0	No	(-)
Ximenia americana (Olacaceae)	(*S*)-*Xa*HNL	Native: 110 SDS : 39-42 (monomer)	3	4.0	5.5	No	Yes
Manihot esculenta (Euphorbi-ceae)	(*S*)-*Me*HNL	Native: 92-124 SDS : 28-30 (heterotrimer or homotetramer)	3	4.1-4.6	5.4	No	No
Hevea brasiliensis (Euphorbi-ceae)	(*S*)-*Hb*HNL	Native: 58-100 SDS : 30±1	(-)	4.1	5.5-6.0	No	No

(-): no reported

Source: Hickel *et al.* (1996).

2.4.6. Substrate specificity

Since the applications of HNLs in chiral cyanohydrins syntheses are much of interest in this decade, the substrate specificity of HNLs was surveyed based on the reaction of asymmetric synthesis of chiral cyanohydrins by using aldehydes or ketones as substrates as summarized in Table 2.

HNLs from Rosaceae family have broad substrate specificity and applicability. (*R*)-HNLs from seeds and kernels of almond, apricot, cherry, plum were used in chiral cyanohydrin syntheses. HNL from apple seed meals accepts sterically hindered aldehydes (e.g. pivaldehyde) as substrates with enantiomeric excess (*e.e.*) better than 90% which was slightly higher than *e.e.* obtained from almond seed meal (Fechter and Griengl, 2004). Loquat seed meal showed the restriction to aromatic and heteroaromatic aldehyde, but gave lower *e.e.* than almond enzyme, while peach meal had the similar substrate range comparing to almond HNL (Lin *et al.*, 1999). HNL isolated from *Linum usitatissimum* has a completely different substrate from that of *Prunus* enzyme. This HNL catalyses the addition of HCN to various aliphatic ketones and aldehydes, while aromatic ketones are not converted. The small steric difference between methyl and ethyl group of 2-butanone is possible to synthesize (*R*)-butan-2-one cyanohydrin (Albrecht *et al.*, 1993). Wajant described the non-FAD containing HNL from *Phlebodium aureum* that the enzyme has no properties in common with the flavoprotein HNLs from Rosaceae, except that it has the same natural substrate, (*R*)-mandelonitrile. This enzyme is suitable for (*R*)-cyanohydrin synthesis in organic media (Wajant *et al.*, 1995).

Table 2. Substrate specificity of hydroxynitrile lyases

Plant	Enzyme source	Stereo-selectivity	Natural substrate	Substrate acceptance	
				Aromatic	Aliphatic
Prunus amygdalus	almond bran	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Prunus serotina	black cherry kernels	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Prunus domestica	plum kernel and leaves	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Prunus avium	cherry kernel and leaves	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Prunus persica	peach kernel and leaves	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Malus pumila	apple seed meal	(*R*)-HNL	(*R*)-mandelonitrile	+	+
Linum usitatissimum	young flax plants	(*R*)-HNL	(R)-2-butanone cyanohydrin	-	+
Phlebodium aureum	goldfoot fern leaves	(*R*)-HNL	(R)-mandelonitrile	+	weak
Pouteria sapota	mamey kernels	(*R*)-HNL	(-)	+	(-)
Cydonia oblonga	quince kernels	(*R*)-HNL	(-)	+	(-)
Cucumis melo	melon seeds	(*R*)-HNL	(-)	+	(-)
Manihot esculenta	cassava leaves	(*S*)-HNL	acetone cyanohydrin	+	+
Hevea brasiliensis	rubber tree leaves	(*S*)-HNL	acetone cyanohydrin	+	+
Sorghum bicolor	millet seedling	(*S*)-HNL	(*S*)-4-hydroxy-mandelonitrie	+	-
Ximenia americana	-	(*S*)-HNL	(*S*)-mandelonitrile	+	-
Annonia muricata	guanabana seed meal	(*S*)-HNL	(-)	+	(-)
Annonia cherimolia	cherimoya seed meal	(*S*)-HNL	(-)	weak	(-)
Annonia squamosa	annona seed meal	(*S*)-HNL	(-)	weak	(-)

(-): no reported

Source: Fechter and Griengl (2004).

(*S*)-HNLs from *Manihot esculenta* and *Hevea brasiliensis* accept the board range substrates of aldehydes and ketones, while HNL from *Sorghum bicolor* is not accepted aliphatic substrates. Recently, the HNL activity has been found in several crude enzyme preparation, for example, *Pouteria sapota, Cydonia oblonga, Cucumis melo, Arabidopsis thaliana, Prunus mume, Chaenomles sinensis, Prunus lyonii, Passiflora edulis, Baliospermum montanum, Annona squamosa, Annona cherimolia, Annona muricata*, etc (Asano *et al.*, 2005; Hernandez *et al.* 2004; Andexer *et al.*, 2007).

3. Cyanohydrins synthesis by hydroxynitrile lyases

Over the last 10 years there has been an explosion of interest in asymmetric cyanohydrin synthesis. The addition of cyanide to carbonyl compounds has a long history. The first chemical synthesis of a cyanohydrin (mandelonitrile) was reported in 1832 by Winkler (North, 2004). The cyanohydrins (Figure 16) establish themselves as key intermediates in organic synthesis. Due to both functional groups, the hydroxyl and the cyanide moiety attached to the same carbon, can be easily converted into a wide range of other chiral products such as α-hydroxy aldehydes and ketones, β-amino alcohols, α-fluoro cyanides, etc. These structures are present in a large number of industrial products such as pharmaceuticals, veterinary products, crop-protecting agents, vitamins and food additives. One possibility for the synthesis of chiral cyanohydrins is the enantioselective addition of HCN to a prochiral aldehyde or ketone with HNLs as biocatalysts (Hickel *et al.*, 1996).

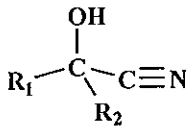


Figure 16. General structure of cyanohydrin.

Source : North (2003).

The first enzyme catalytic asymmetric cyanohydrin synthesis was reported in 1908 (Rosenthaler, 1908). The extract of defatted almonds (emulsion) would catalyse the asymmetric addition of HCN to aldehyde giving (*R*)-cyanohydrin. HNL from almonds (*Prunus*

amygdalus) was purified and reported to be a catalyst for cyanohydrins synthesis (Becker *et al.*, 1963). After that, several (*R*)- and (*S*)-HNLs were discovered and used wildly in the cyanohydrins synthesis. Some chemical structures and applications of cyanohydrins derivatives are presented in Table 3.

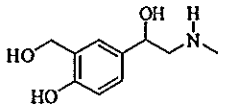
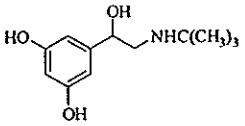
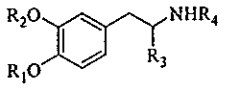
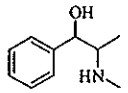
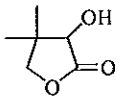
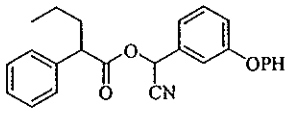
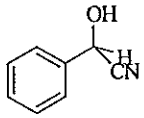
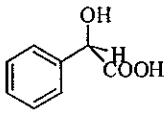
3.1. Cyanohydrins synthesis systems

Enantiomeric syntheses of cyanohydrin are affected by the non-enzymatically spontaneous chemical reaction. The spontaneous addition of HCN to aldehydes or ketones yield racemic of the products which is not desired. Although HNLs were employed in the cyanohydrin synthesis, the spontaneous reaction still parallel occurs and causes of the low enantiomeric excess (*e.e.*). Therefore, several synthesis systems were investigated to suppress the non-enzymatic reaction and obtain the high *e.e.* of the desired products. The *e.e.* of target chiral compounds of 98% is a minimum acceptable level for commercial process (Pollard and Woodley, 2006).

The cyanohydrin synthesis reaction can be run in aqueous buffer, but the problem during the synthesis of enantiopure cyanohydrins in aqueous systems are the formation of racemic compounds of chiral cyanohydrins by non-enzymatic reaction and the low solubility of hydrophobic substrates and products. Therefore, reaction of HNL was carried out in buffer at low pH (pH 3.5-4.0) (Schmidt *et al.*, 1996) and temperature (0-5 °C) (Griengl *et al.*, 1997) to suppress the non-enzymatic reaction and increase the *e.e.* of the product. However, at low pH the stability of the enzyme was affected significantly and the activity of the enzyme was reduced in the case of *Hb*HNL. The excess amount of enzyme in the reaction was used to solve this problem (Hickel *et al.*, 1997).

The mixture of buffer and water-miscible solvent was applied for cyanohydrin synthesis by HNLs. In aqueous/alcoholic buffer system, the *e.e.* of product decreases significantly because the non-enzymatic reaction is still a problem (Sharma *et al.*, 2005). Alternatively, the use of HNLs in biphasic system of buffer and immiscible organic solvent provides many advantages. The principle is to combine optimum enzyme performance in the buffered aqueous phase with a high productivity by employing the organic phase as a reservoir of both substrate and product.

Table 3. Chemical structure and application of some cyanohydrin derivatives.

No.	Cyanohydrin derivative	Chemical structure	Application
1	(*R*)-Salbutamol	 (*R*)-salbutamol	Bronchodilator
2	(*R*)-Terbutaline	 (*R*)-terbutaline	Bronchodilator
3	(*S*)-Amphetamines	 (*S*)-amphetamine	Designer drug used medically in psychological treatments and central nervous stimulant
4	L-Ephedrine	 (1*R*,2*S*)-(-)-ephedrine	Stimulant and bronchodilator
5	(*R*)-Pantolactone	 (*R*)-pantolactone	Chiral building block and chiral auxiliaries, constituents of coenzyme A, bactericide, A growth factor
6	Pyrethroids	 Fenvalcrate	Insecticide
7	Mandelonitrile	 Mandelonitrile	Germicide, pemoline
8	Mandelic marine complexes	 Mandelic acid	Cosmetics

Soruce : Sharma *et al.* (2005).

The benefits in using biphasic system are highly efficient, and cost effective production method due to increase of substrate concentration, high product yield, easy down stream processing as enzyme remain partitioned to aqueous phase, and it is not necessary to immobilize the enzyme. However, in the biphasic system, a number of reaction parameters are involved in a complex interplay to yield the *e.e.* and conversion for the reaction (Sharma *et al.*, 2005; Griengl *et al.*, 1997).

For practical application of HNLs as catalysts for the preparation of chiral cyanohydrins, three objectives have to be achieved: first, to get high enantioselectivity by suppress the non-enzymatic addition of HCN to the substrate. Secondly, especially for industrial application high reaction rates are desirable for efficient large-scale production. Thirdly, *in situ* formation of HCN is favorable to avoid free handling of toxic HCN (Effenberger *et al.*, 2007).

3.2. Parameters influencing on chiral cyanohydrin synthesis by hydroxynitrile lyases in biphasic system

Asymmetric syntheses of chiral cyanohydrins in the biphasic system are influenced by buffer pH, reaction temperature, organic solvent, aqueous phase content, and cyanide source during the catalysis of HNLs.

3.2.1. pH

pH is an important parameter to control the enantioselectivity of enzymes to achieve the high enantiomeric excess of their products. The effect of pH on the enantioselectivity was observed on several enzymes such as hydrolase, lipase, etc (Lo´pez-Serrano *et al.*, 2001; Sakai, 2004; Wang and Tsai, 2005).

Syntheses of chiral cyanohydrins by using HNLs as biocatalysts are also influenced by reaction pH. The non-enzymatic reaction of cyanohydrins synthesis is prone to occur rapidly when increase the pH. On the synthesis of cyanohydrins by *Hevea brasiliensis, Manihot esculenta, Sorghum bicolor, and Prunus amygdalus*, high *e.e.* of the products was obtain when performed the reaction at low pH (Costes *et al.*, 1999; Presson *et al.*, 2002).

3.2.2. Temperature

Temperature also influenced on the progress of the reaction and *e.e.* of cyanohydrin products. The enzyme stability and enantiomeric product yield is enhanced at low temperature (Sharma *et al.*, 2005). Increase of temperature accelerate the non-enzymactic reaction and cause the low in *e.e.* of product (Costes *et al.*, 1999; Presson *et al.*, 2002). At temperature below 5 °C, the stability of the enzyme against denaturation is enhanced and it is sufficient to obtain the corresponding chiral cyanohydrin in good yield and enantiomeric purity (Griengl *et al.*, 1997).

3.2.3. Organic solvents

Most of solvents used in biphasic system are ethyl acetate, diisopropyl ether and other ethers. Previous studies shown that while using biphasic system the enzyme activity was at the interface rather than in one phase of the biphasic system (Kiljunen and Kanerva, 1997). *Hb*HNL in diisopropyl ether/aqueous interface was more active than in ethyl acetate (Hickel *et al.*, 1999). *Pa*HNL in diisopropyl ether is retained enzyme activity for several weeks. In contrast to ethyl acetate, the *Pa*HNL activity was reduced by greater than 50% in less than 6 h (Johnson and Griengl, 1999).

Partition of substrates between the organic and aqueous phases has a pronounce influence on the enantiomeric purity of the chiral product. The substrate that partition towards the aqueous phase yields products with significantly decreased enantiomeric purity. Conversely, substrates with low water solubility and dissolved in organic phase yield the almost emantiomerically pure products. This trend was also reflected in the values of the logarithm of the partition coefficients (log *P*). For those with positive log *P*, high *e.e.* (88-99%) for the products were recorded, while negative log *P* yielded the product cyanohydrin with low *e.e.* (2-30%) (Ognyanov *et al.*, 1991). This finding is crucial to the successful production of cyanohydrins in this biphasic system.

3.2.4. Aqueous phase content

Water is one of important factors in the use of enzymes in organic solvent. Water is essential parameter for the catalytic activity and stability of enzymes. Nevertheless, the cyanohydrin synthesis process was affected by the aqueous phase content in the reaction. Increasing of aqueous phase content caused the high rate of non-enzymatic reaction and low in *e.e.* of the cyanohydrin products, conversely the absence of moisture or water in the reaction, catalytic activity of HNL was reduced to zero (Presson *et al.*, 2002).

3.2.5. Cyanide source

Hydrogen cyanide (HCN) might be the most preferred cyanide source in cyanohydrin synthesis (Zanbergen *et al.*, 1991). Besides HCN, several cyanide sources e.g. potassium cyanide, sodium cyanide have been used in the synthesis of cyanohydrin. These cyanide sources act as an effective source of cyanide in the low pH range of buffer. The spontaneous addition of HCN might cause the reduction of enantiomeric purity of the product because the high concentration of HCN may increase the non-enzymatic reaction and the directly addition of HCN might cause insoluble of the compound and lead to its immediately release from reaction mixture (Sharma *et al.*, 2005). Alternatively, the indirect generation of HCN was introduced by addition of the acid to the aqueous solution of alkali cyanide (e.g. the addition of acetic acid to the solution of sodium cyanide) and the transcyanation process (Effenberger *et al.*, 2007). This slow diffusion of HCN gives advantage over spontaneous addition and results in high enantiomeric purity of product.

4. Transcyanation of cyanohydrins

Since HCN is highly toxic reagent in direct handling and generated for using in cyanohydrin synthesis, transcyanation was introduced to solve this problem (Ognyanov *et al.*, 1991). Acetone cyanohydrin was a choice of cyanide donor that proves to be advantageous for a number of reasons. It is miscible with buffer used in the system, commercially available, and its by-product acetone is volatile and has a favorable equilibrium constant.

In a recent development, the kinetics of the transcyanation process between benzaldehyde and acetone cyanohydrin were studied by ^{1}H-NMR methods to further elucidation the mechanism of the HCN exchange process. Thus, during a time course study, the quantities of each component in the reaction (i.e. mandelonitrile, benzaldehyde, acetone cyanohydrin and acetone) were quantified. It has been concluded that the transcyanation consists of two separate steps which are cleavage of acetone cyanohydrin followed by synthesis of the new cyanohydrin as shown in Figure 17 (Hanefeld *et al.*, 1999; Johnson and Griengl, 1999).

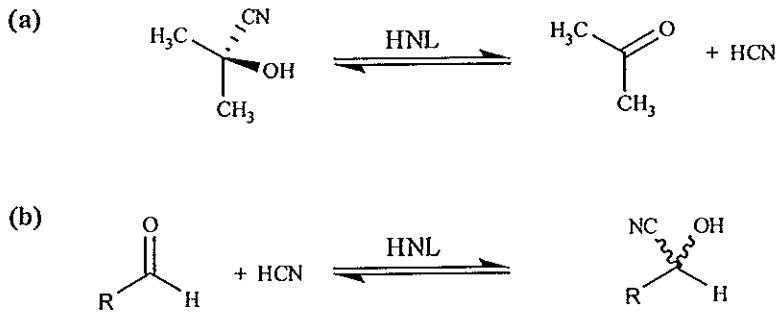


Figure 17. The two independent steps of the transcyanation reaction catalyzed by hydroxynitrile lyase.

Source: Hanefeld *et al.* (1999).

5. Synthetic potential of chiral cyanohydrins in stereoselective synthesis

Chiral cyanohydrins are valuable versatile building blocks because they can be used to create tailored molecules and have large number of applications in chemical industries. These cyanohydrins serve as intermediate substrates that can be further converted into valuable products. Chiral cyanohydrins can be transformed into variety of products using chemical reactions such as reaction of cyano group (Figure 18), reaction of hydroxyl group, reaction of (*R*)-substituents, conversion of *O*-protected cyanohydrins (Figure 19), and conversion of hydroxyl group to a good leaving group to allow nucleophilic displacement with inversion of configuration (Figure 20) (Effenberger *et al.*, 2007).

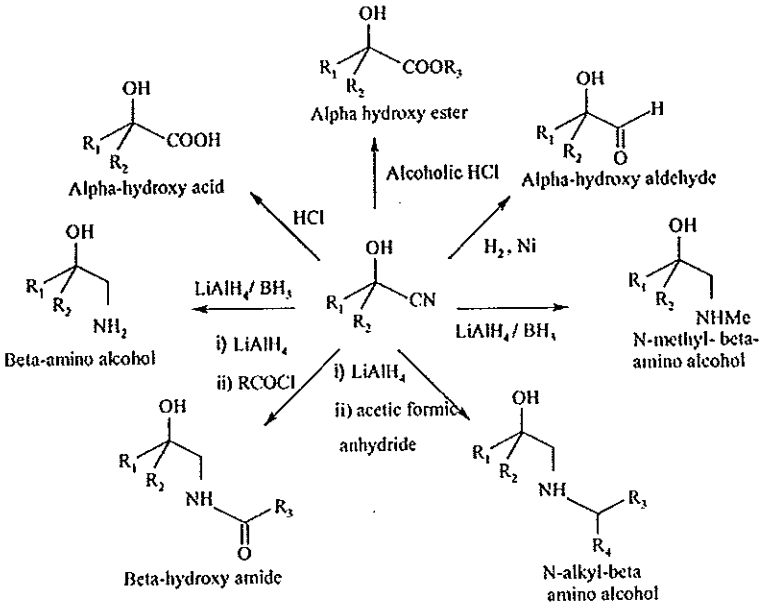


Figure 18. Cyano group transformation of cyanohydrins

Source: Sharma *et al.* (2005).

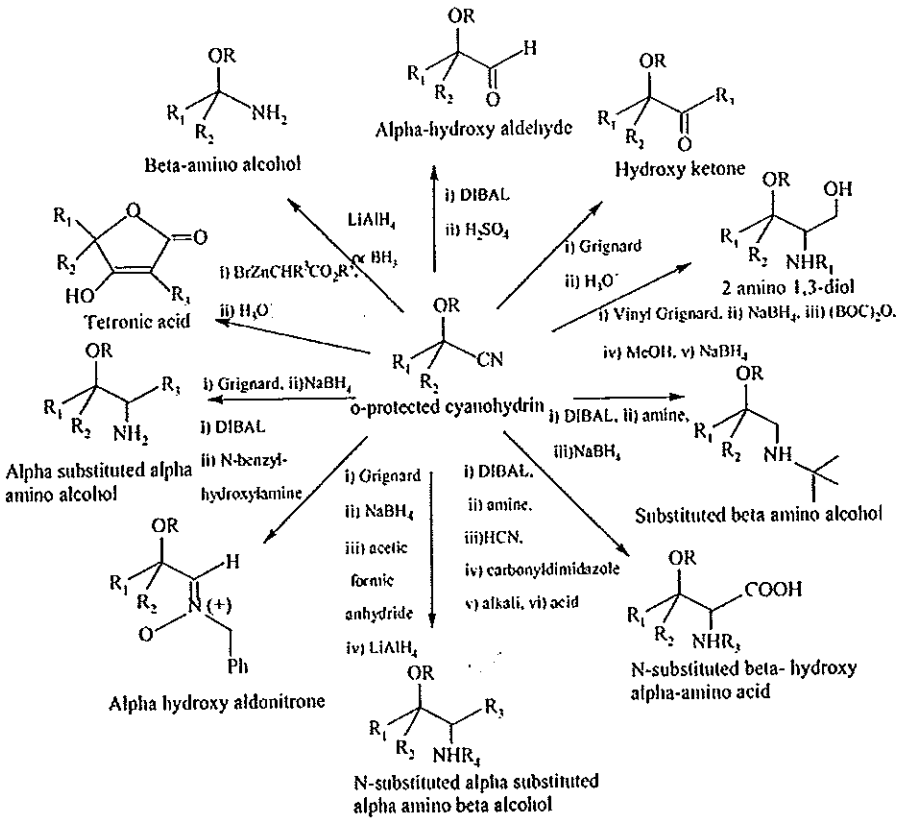


Figure 19. *O*-protected group transformation of cyanohydrins

Source: Sharma *et al.* (2005).

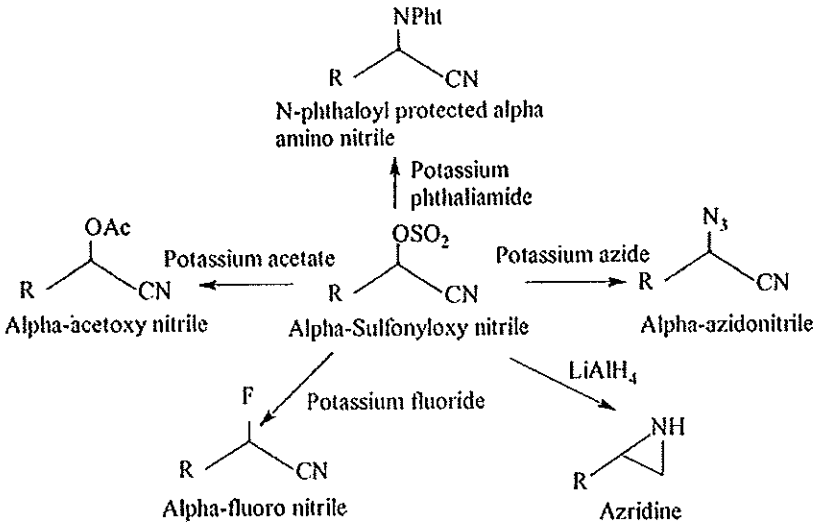


Figure 20. Conversion of good leaving group transformation of cyanohydrins

Source: Sharma *et al.* (2005).

6. (*R*)-Mandelonitrile

(*R*)-Mandelonitrile is the cyanohydrin derived from benzaldehyde. Naturally occurring of (*R*)-mandelonitrile is found as an intermediate of cyanogenesis in higher plants. Since the (*R*)-mandelonitrile is one of important chiral cyanohydrins with can be converted to several valuable commercial products, the synthesis of (*R*)-mandelonitrile is attracted the interests of scientists and industries. One possibility to synthesis of (*R*)-mandelonitrile is to employ the enantioselective addition of (*R*)-hydroyxnitrile lyase by using benzaldehyde and cyanide as described elsewhere (Johnson and Griengl, 1999; Kruse, 1992). In this section, some of the products derived from (*R*)-mandelonitrile and their application are demonstrated.

6.1. Ephedrine

Ephedrine (l-ephedrine, (1*R*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol) is an alkaloid derived from various plants in the genus *Ephedra* (Family Ephedraceae). Ephedrine (Figure 21) is similar in structure to the synthetic derivatives of amphetamine and methamphetamine. It is used clinically to prevent bronchospasm (a sudden constriction of the

muscles in the walls of the bronchioles causes difficulty in breathing) during surgical procedures, to treat acute hypotension (abnormal low blood pressure), and as a nasal decongestant (a drug that shrinks the swollen membranes in the nose, making it easier to breath) (Rorabaugh, 2007).

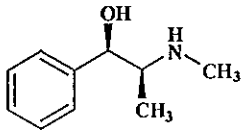


Figure 21. Structure of ephedrine.

Source: Rorabaugh (2007).

6.2. Pemoline

Pemoline (2-imino-5-phenyl-4-oxazolidinone) has been used in the treatment of hyperactivity disorders and other behavioral syndromes (Erhorn, 2007). The structure of pemoline is shown in Figure 22.

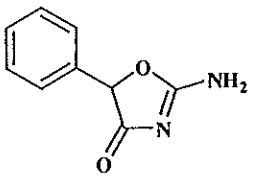


Figure 22. Structure of pemoline.

Source: Erhorn (2007).

6.3. (*R*)-Mandelic acid

Among α-hydroxy acids derivative of chiral cyanohydrins, optically active 'mandelic acids' are regarded as most important commercially (Groger, 2001). Chemical reactions on the nitrile group of (*R*)-mandelonitrile can be carried out without the need of protection is acid-catalysed hydrolysis. Hydrolysis of (*R*)-mandelonitrile give the corresponding (*R*)-α-mandelic acid in excellent yields with high enantiomeric purity (Groger, 2001).

(*R*)-Mandelic acid (Figure 23) is a chiral building block for the production of antiaging agents, antiobesity agents, antitumor agents, penicillins, urine antiseptic, etc., and is also used as a chiral resolving agent. Production of (*R*)-mandelic acid can be achieved by chemical as well as by enzymatic routes (He *et al.*, 2007; Herold, *et al.*, 2002).

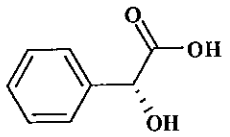


Figure 23. Structure of (*R*)-mandelic acid.

Source: Kruse (1992).

6.4. Mandelic acid condensation product (SAMMA)

Recently, mandelic condensation product (SAMMA, a polymer derived from sulfuric acid treatment of mandelic acid), has been identified as a candidate topical contraceptive (developed by the Topical Prevention for Conception and Disease, Rush University, Chicago). In preliminary work, SAMMA exhibited an excellent safety profile and was active against HIV, HSV, bovine papillomavirus, and *Neisseria gonorrhoeae*. SAMMA inhibits HIV infection of both primary CD4+ T cells and macrophages. Importantly, SAMMA has no deleterious effects on lactobacilli. Its simple chemical structure, solubility in water, apparent stability, and characteristics of being colorless and odorless make it attractive as a candidate microbicide. Moreover unlike other compounds being developed as topical microbicides, it does not contain sulfur, and it is not sulfated or sulfonated, and is not a surfactant (Herold, *et al.*, 2002).

Objectives

HNLs and their applications in asymmetric synthesis of chiral cyanohydrins have been the subject of much interest among the scientists and industrial applications for many years. However, the demand for new HNLs and improvement of chiral cyanohydrin synthesis approach remain desirable.

The first objective of this research was to investigate the occurrence of cyanogenesis and the present of HNLs in plants especially in Thai plants. Since many Thai plants are members of cyanogenic plant families and there is less information of cyanogenesis in Thai plants. Therefore, several new cyanogenic plant species were investigated by using picrate method and reported in this research. Furthermore, the survey of cyanogenesis plants opened the door to discover the novel HNLs which involves in the cyanogenesis.

The second objective aimed to isolate, purify the HNL from plant. The HNL from *Eriobotrya japonica* (loquat) was purified to homogeneity. The characteristics of the enzyme including molecular mass, *N*-terminal amino acid sequence, optimum pH and temperature, stability on pH and temperature, inhibitors were investigated. Moreover, the substrate specificity of the enzyme on cyanohydrins synthesis and the enantiomeric excess of the cyanohydrin products were determined in aqueous phase system.

For practical application of HNL in cyanohydrin synthesis, (*R*)-mandelonitrile was selected for synthesis due to the board application of the compounds. The synthesis was studied and optimized to achieve the highest enantioselectivity and reaction rate. Furthermore, transcyanation reaction employing acetone cyanohydrin as a cyanide source was carried out along the study to avoid the direct handling of hydrocyanic acid.

The third objective was to study on the parameters influencing on asymmetric synthesis of (*R*)-mandelonitrile by HNL from *E. japonica* in biphasic systems. Since several parameters are influenced on the enantiomeric excess of cyanohydrin products, the essential parameters such as pH, temperature, organic solvents, aqueous phase content, substrates and enzyme concentration were optimized to achieve the highest enantiomeric excess of (*R*)-mandelonitrile product and reaction velocity.

The last objective was to study and optimize the parameters influencing on asymmetric synthesis of (*R*)-mandelonitrile by HNL isolated from *Passiflora edulis* (passion fruit) in biphasic system. Several parameters have been optimized in order to obtain the highest enantiomeric excess of (*R*)-mandelonitrile and reaction velocity by transcyanation reaction.

CHAPTER 2

The occurrence of cyanogenesis and hydroxynitrile lyase in some Thai plants

2.1 Abstract

The leaves, seeds, fruits and shoots of 176 plant species in 26 families of Thailand were screened for the presence of cyanide (HCN) using the picrate paper test. The known cyanogenic plants and their cyanide content were observed in 11 species which were leaves of *Lisia spinosa* (1,258 ppm), leaves of *Acalypha indica* (1,020 ppm), leaves of *Elateriospermum tapos* (7 ppm), leaves of *Hevea brasiliensis* (2,961 ppm), leaves of *Manihot esculenta* (1,013 ppm), young shoot of *Bambusa bambos* (1,822 ppm), leaf of *Acacia farnesiana* (4 ppm), leaves of *Dalbergia cochinchinensis* (1,795 ppm), young leaves of *Passiflora cocinea* (833 ppm), leaves of *Passiflora edulis* (1,085 ppm), and leaves of *Passiflora quadrangularis* (1,169 ppm). The new sources of cyanogenic plants and their cyanide contents were investigated in 5 species which were leaves of *Afzelia xylocarpus* (1,329 ppm), seedling of *Archidendron jiringa* (17 ppm), leaves of *Dalbergia floribunda* (625 ppm), leaves of *Xantosoma nigrum* (15 ppm), and leaves of *Hydnocarpus ilicifolia* (707 ppm). The increase in pH and temperature accelerated the decomposition of cyanohydrin (mandelonitrile) significantly. The (*S*)-Hydroxynitrile lyases (HNLs) activity presented in leaves of *Hevea brasiliensis* (3.28 U/mg) and *Manihot esculenta* (2.13 U/mg), while (*R*)-HNL activity was investigated in leaves, seeds, and rind of *Passiflora edulis* with specific activity of 1.47, 1.23, and 0.92 U/mg. The (*R*)-HNL from *P. edulis* showed ability in cyanohydrin synthesis with enantiomeric excess of 54.6, 47.9, and 33.2% in the enzyme extracted from leaves, seeds, and rind of plant, respectively.

2.2 Introduction

Cyanogenesis is the ability of living organisms to produce hydrogen cyanide (HCN). It is widely distributed in higher plants (Spermatophyta). At least 2,650 plant species produce sufficient quantities of cyanogenic compounds and form the detectable quantities of HCN, including some important food plants like cassava and sorghum (Conn, 1980a; Aikman *et al.*, 1996; Haque and Bradbury, 2002; Jones, 1998, Kake, 1991; Lewis and Zona, 2000; Maslin *et al.*, 1988; Vetter, 2000).

HCN is extremely toxic to a wide spectrum of organisms. Some plants produce hydrogen cyanide to protect themselves against predators and microorganisms (Hickel *et al.*, 1996). The acute toxicity of HCN is due to its affinity to forming various heavy metals cyano-complexes such as iron (Fe^{++}), copper (Cu^{++}) and manganese (Mn^{++}). These complexes might inhibit the activity of enzymes in respiratory chain (i.e. cytochrome oxidases) and other enzymes containing heavy metal ions (Francisco and Pinotti, 2000; Gleadow *et al.*, 2003; Selmar, 1999). There are several methods for the quantitative analysis of HCN (Alonso-Amelot and Oliveros, 2000). Picrate method has been widely used to survey for cyanogenesis in the field, since it is very simple to use, accurate, and reproducible (Bradbury *et al.*, 1999; Egan *et al.*, 1998).

In cyanogenesis, HCN will be released by action of enzymes in the plant acting on the cyanogenic glycosides when the cellular structures of plant tissues are destroyed (Conn, 1980; McMahon *et al.*, 1995). The initial step of cyanogenesis is hydrolysis of cyanogenic glycosides to cyanohydrin or α-hydroxynitrile and glucose by β-glucosidase. Subsequently, the unstable cyanohydrin decomposes to HCN and the corresponding carbonyl compounds spontaneously or by the action of hydroxynitrile lyases (HNLs) (Figure 24) (McMahon *et al.*, 1995). As the enzymatic reactions are reversible, HNLs can also be used for the synthesis of enantiomerically pure cyanohydrins which are valuable structures for the chemical synthesis in pharmaceuticals, agrochemicals and fine chemicals industries (Hernandex *et al.*, 2004; Wajant and Effenberger, 1996).

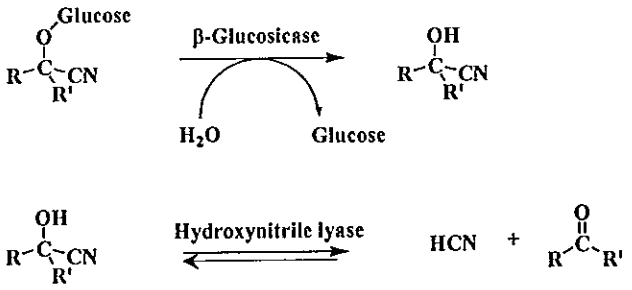


Figure 24. Initial step of cyanogenesis by enzymatic catalysis and catalytic reaction of hydroxynitrile lyase.

Source: McMahon *et al.* (1995).

Since HNLs are of interested as valuable biocatlysts for synthesis of the highly pure enantiomeric chiral cyanohydrin. The number of plant species containing HNLs found up to date was only 26 species (Hickel *et al.*, 1996; Yemm and Poulton, 1986; Hickel *et al.*, 1997; Albrecht *et al.*, 1993; Wajant and Forster, 1996; Wajant *et al.*, 1995; Kuroki and Conn, 1989; Jansen *et al.*, 1992; Hughes *et al.*, 1994; Asano *et al.*, 2005), comparing with the number of cyanogenic plant species (2,650 species) is still small. Therefore, the relationship of the occurrence of cyanogenesis and the presence of HNLs in cyanogenic plants in Thailand with intention of discovering new HNL activity was investigated in this present study.

2.3 Materials and methods

2.3.1 Plant materials

The plant samples were purchased or collected from 5 areas in Thailand from January to August 2004: a) Fresh market in Hat Yai; b) Peninsular Botanic Garden, Thung Khai, Trang; c) Prince of Songkla University, Hat Yai; d) Southern Flora with Its Thai Literature Collection, Hat Yai and e) Queen Sirikit Botanic Garden, Chiangmai. Tests were conducted mostly with either young shoots, leaves or seeds. Most plants were harvested in the morning and tested in the afternoon in the same day.

2.3.2 Screening of cyanogenic plants

The screening of cyanogenic plant was carried out using picrate paper test according to the methods of Francisco and Pinotti (2000), Gilchrit *et al.* (1967) and Egan *et al.* (1998) with slight modification. Plant samples (5 g) were ground in liquid nitrogen by pestle and mortar (Figure 25a), and placed in screw cap test tube. Distilled water (1.5 ml) and $CHCl_3$ (5 drops) were added to the test tube then the strip of moisten freshly prepared sodium picrate paper was suspended over the crushed material. The test tube was immediately tightened and incubated at 30 °C for 24 h. The change in color of picrate paper from yellow to orange/red showed an occurrence of HCN liberated from crushed plant materials (Figure 25b). Leafs of *Manihot esculenta* Crantz, was used as positive control.

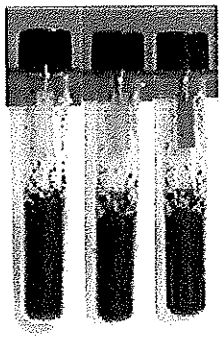
(a)  (b) 

Figure 25. Screening of cyanogenic plant by picrate paper test. (a) Ground plant sample in liquid nitrogen by pestle and mortar. (b) The change in color of picrate paper from yellow to orange/red showed occurrence of HCN liberated from crushed plant materials.

2.3.3 Determination of cyanide content using picrate paper test

The cyanide content in cyanogenic plants was determined by using picrate paper test as in 1.3.2. After 24 h, the strip of picrate paper was removed from the test tube, immersed in 5 ml of distilled water, shaked for 30 min. The intensity of the solution was measured by

spectrophotometer at 510 nm. The picrate paper developed in the absence of cyanogenic plant was used as blank

Standard curve of cyanide content was prepared according to the method of Egan *et al.* (1998) with slight modification. The stock cyanide solution (0.0037 M KCN), giving range from 1-10 µg HCN, was added to the screw cap test tube. Then 5 ml of 5 M sulphuric acid was added, followed immediately by suspension of moisten picrate paper and tighten the test tube. The color development of picrate papers was followed in experiment for 24 h. Strip of picrate paper was immersed in 5 ml of distilled water, shaked for 30 min and the adsorbance was measured at 510 nm. Blank was prepared as describe above without cyanide solution.

2.3.4 Effect of pH and temperature on the stability of mandelonitrile

The decomposition of mandelonitrile was measured by monitoring the decomposition of (*R,S*)-mandelonitrile to benzaldehyde by continuously measuring the increase in absorbance at 280 nm. The reaction was performed in a quartz cell.

The reaction contained 2 mM mandelonitrile in 50 mM sodium citrate phosphate buffer in a total volume of 1 ml at various pH and temperature. Then, the reaction was mixed gently and the reaction was followed for 1 min by spectrophotometer at 280 nm (U-3210 spectrophotometer, Hitachi, Japan). By using ε_{280}=1.4 $mM^{-1}cm^{-1}$, the concentration of benzaldehyde decomposes from mandelonitrile was calculated.

2.3.5 Screening of hydroxynitrile lyase

2.3.5.1 Preparation of the crude enzyme extract

The cyanogenic plants were ground in liquid nitrogen by using pestle and mortar. Ground plant sample (5 g) was extracted on with 10 ml of 10 mM potassium phosphate buffer, pH 6.0 and stirred for 2 h at 4 °C. The solution was filtered and squeezed through layers of cheesecloth. Filtrate was centrifuged at 28,000*g* and 4 °C for 30 min. The supernatant was used as crude enzyme extract and assayed for the enzyme activity.

2.3.5.2 HNLs assay

The HNL activity was assayed according to the method of Asano *et al.* (2005) with slight modification. The enzyme activity was assayed by measuring the production of optically active mandelonitrile synthesized from benzaldehyde and cyanide.

A reaction mixture in a total volume of 1.0 ml was prepared in a micro-tube. Benzaldehyde (1.0 M in DMSO, 40 µl) was added to sodium citrate buffer (400 mM, pH 4.0), followed by the enzyme solution and a KCN solution (1.0 M, 100 µl). The initial velocity of the reaction was monitored by taking a small aliquot of the reaction mixture (100 µl) and the reaction was stopped by extracting with 700 µl 85% *n*-hexane and 15% isopropanol by volume. The mandelonitrile formed was extracted into the organic layer, and obtained by centrifugation at 15,000 g for 1 min at 4 °C was analyzed by HPLC with a CHIRALCEL OJ-H column at 254 nm using 85% *n*-hexane and 15% isopropanol by volume as a mobile phase at a flow rate of 1.0 ml/min. The retention times of benzaldehyde and (*R*)- and (*S*)-mandelonitrile were about 4.9, 10.2, and 12.7 min, respectively. The reaction progressed linearly in the first five minutes was used for calculating activity.

One unit of HNL activity was defined as the amount of enzyme that produced 1 µmol of optically active mandelonitrile from benzaldehyde per min under standard assay conditions.

2.3.6 Protein assay

Protein concentration of crude HNL extracts was determined according to the method of Lowry *et al.* (1951) using a bovine serum albumin as a standard.

2.4 Results

2.4.1 The occurrence of cyanogenesis in some plant species

The leaves, seeds, fruits and shoots of 176 plant species in 26 families were tested for the presence of cyanide (HCN) using the picrate paper test. The results are shown in Table 4.

One plant species in Anacardiaceae, 10 species in Annonaceae, 2 species in Apocynaceae, 1 species in Araliaceae, 1 species in Basellaceae, 15 species in Bignoniaceae, 1 species in Boraginaceae, 6 species in Capparaceae, 1 species in Caricaceae, 2 species in Compositae, 1 species in Dioscoreaceae, 1 species in Laeaceae, 1 species in Lauraceae, 1 species in Menispermaceae, 2 species in Myrtaceae, 1 species in Poteaceae, 6 species in Rosaceae and 1 species in Sapindaceae were tested but no cyanogenic activity was detected.

Two of 8 plant species in Araceae, leaves of *Lisia spinosa* and leaves of *Xantosoma nigrum*, performed cyanogenic activity. The HCN contents were 1,258 and 15 ppm, respectively.

Afzelia xylocarpus, belong to Caesalpinoideae, was cyanogenic. The cyanide content in leaves of this plant was 1,328 ppm, but other 30 species in this family were acyanogenic plants.

In family Euphorbiaceae, the content of cyanogenic glycosides and the ability of releasing HCN in this family differ greatly depending on species or variety of plants (Berg *et al.*, 1995; Lieberei *et al.*, 1986; Selmar *et al.*, 1987; Selmar *et al.*, 1991). The 40 plant species in this family were subjected to screen for cyanogenesis. The presence of cyanogenesis was observed in 4 species. Leaves of *Acalypha indica* L. showed strong cyanide content (1,020 ppm). As well as in *Hevea brasiliensis* and *Manihot esculenta* Crantz, the cyanide content in leaves of both plants was 2,961 and 1,013 ppm, respectively. Generally, the mature leaves of *Manihot esculenta* Crantz have large amount of cyanide content from 900-2,000 ppm (Bokanga, 1994) whereas the cyanide content in fresh *Hevea* seeds varied widely about 2,830 ± 1,423 ppm (Lieberei *et al.*, 1986). Contrary to leaves of *Elateriospermum tapos* had small amount of cyanide content (7 ppm).

Three plant species in Flacourtiaceae was selected for screening, but only leaves of *Hydnocarpus ilicifolia* was a strong cyanogenic plant. The fresh leaves of *H. ilicifolia* liberated 707 ppm of HCN. In addition, the occurrence of cyanogenesis in Flacourtiaceae has been found in *Rawsonia lucida*, *Gynocardia odorata*, *Pangium edule*, *Carpotroche brasiliensis*, *Hydnocarpus anthelmintica* and *Caloncoba echinata* (Andersen *et al.*, 2001).

Cyanogenesis was investigated in Gramineae. The bamboo shoots of *Bambusa bambos* gave cyanide content of 1,822 ppm, while *Zea mays* was acyanogenic plant. Previous study reported that *Bambusa arundinaceae* gave cyanide content of 100-1,600 ppm (Haque and Bradbury, 2002).

In family Mimosoideae, 19 plant species were used for screening. *Acacia farnesiana* showed small amount of cyanide content in leaves (4 ppm). Cyanogenesis in leaves of *A. farnesiana* has been reported to liberated cyanide 0-5.5 ppm and the cyanogen present was elucidated as linamarin and lotaustralin (Janzen *et al.*, 1980; Seigler *et al.*, 1979). There was no cyanide liberation in ground seed and leaves of *Archidendron jiringa* but seedling after germination of *A. jiringa* gave small amount of cyanide content (17 ppm). There are several reports on changing of cyanogenic potential during germination, seedling development and subsequent growth of plants. As seeds of *A. jiringa*, the cyanogenic potential of *Sorghum, Lotus* and *Linum* seeds were slightly increased during germination (Niedźwiedź-Siegień, 1998; Haque and Bradbury, 2002).

Table 4. Screening of cyanogenic plants and their cyanide contents.

Family	Plant species	Part	HCN content (ppm)	Source
Anacardiaceae	*Anacardium occidentale* L.	leaf/seed/fruit	0	a
Annonaceae	*Annona muricata* L.	leaf	0	e
Annonaceae	*Annona squamosa*	seed	0	a
Annonaceae	*Artabotrys hesapetalus* (Linn. f.) Bhandan	young leaf/leaf	0	e
Annonaceae	*Cananga odorata* (Lam.) Hook. f.& Thomson	leaf	0	e
Annonaceae	*Friesodielsia desmoids* Steenis	leaf	0	e
Annonaceae	*Polyathia viridis* Craib	young leaf/leaf	0	e
Annonaceae	*Rauwenhoffia siamensis* Scheff.	leaf	0	e
Annonaceae	*Uvaria grandiflora* var. flora Sincl.	leaf	0	e
Apocynaceae	*Ichnocarpus batatas* (L.) Lam.	root	0	a
Apocynaceae	*Thevetia peruviana* (Pers.) K.Schum	leaf/fruit	0	c
Araceae	*Alocasia macrorrhizos* (L.) G.Don	leaf	0	c
Araceae	*Amorphophallus boycei* Hett.	leaf	0	c
Araceae	*Colocasia esculenta* (L.) Schott	root	0	a
Araceae	*Epipremnum giganteum* (Roxb.) Schott.	leaf	0	d
Araceae	*Homalomena rubescens*-Kunth	leaf	0	c
Araceae	*Lisia spinosa* (L.) Thwaites.	leaf	1,258	b
Araceae	*Spathishyllum* sp.	leaf	0	e
Araceae	*Xantosoma nigrum* (veil.) Mansf.	leaf	15	c
Araliaceae	*Schefflera leucantha* R. Vig.	leaf	0	c
Basellaceae	*Anredera cordifolia* (Ten.) Steenis	seed/leaf	0	a
Bignoniaceae	*Crescentia cujete* L.	leaf	0	d
Bignoniaceae	*Dolichandrone serrulata* (DC.) Seem.	leaf	0	a
Bignoniaceae	*Fernandoa adenophylla* Steenis	leaf	0	c
Bignoniaceae	*Markhamia stipulate* Seem.	leaf	0	e
Bignoniaceae	*Millingtonia hortensis* Linn. f.	leaf	0	e
Bignoniaceae	*Oroxylum indicum* Vent	leaf	0	c
Bignoniaceae	*Pachyptera hymenaea* A. Gentry.	young leaf/leaf	0	e
Bignoniaceae	*Pauldopia ghorta* (G. Don) Steenis	leaf	0	e
Bignoniaceae	*Pyrostegia venusa* Miers	leaf	0	e
Bignoniaceae	*Radermachera ignea* Steenis	young leaf/leaf	0	e
Bignoniaceae	*Radermachera glandulosa* (Blume) Miq.	leaf	0	c

Table 4. (Continued)

Family	Plant species	Part	HCN content (ppm)	Source
Bignoniaceae	*Spathodea campanulata* Beauv.	leaf	0	c
Bignoniaceae	*Tabebuia chrysantha* (Jacq.) G. Nicholson	leaf	0	d
Bignoniaceae	*Tecoma stans* (L.) Kunth	leaf	0	c
Bignoniaceae	*Tecomaria capensis*	leaf	0	e
Boraginaceae	*Tournefortia ovata* Wall. ex G.Don	seedling	0	a
Caesalpinioideae	*Afzelia xylocarpus* (Kurz.) Craib.	leaf	1,329	d
Caesalpiniodeae	*Bauhinia acuminate* Linn.	leaf/seed	0	c
Caesalpiniodeae	*Bauhinia binata* Blanco	leaf	0	c
Caesalpiniodeae	*Bauhinia glauca* ssp. Tenuiflora K.&S. Larsen	leaf	0	e
Caesalpiniodeae	*Bauhinia monandra* Kurz	leaf/seed	0	c
Caesalpinioideae	*Bauhinia purpurea* Linn.	leaf/seed	0	c
Caesalpiniodeae	*Bauhinia scandens* Linn.	leaf	0	c
Caesalpiniodeae	*Bauhinia variegate* Linn.	leaf	0	e
Caesalpiniodeae	*Brownea ariza* Benth	leaf	0	c
Caesalpiniodeae	*Caesalpinia coriaria* Willd.	leaf	0	c
Caesalpinioideae	*Caesalpinia crista* Linn.	leaf	0	c
Caesalpinioideae	*Caesalpinia pulcherrima* (L.) SW.	leaf/seed	0	c
Caesalpinioideae	*Caesalpinia sappan* Linn.	leaf/seed	0	c
Caesalpiniodeae	*Cassia bakeriana* Craib	leaf	0	c
Caesalpinioideae	*Cassia fistula* Linn.	leaf	0	c
Caesalpinioideae	*Cassia siamea* Lam.	leaf	0	e
Caesalpinioideae	*Dialium cochinchinese* Pierre	leaf	0	c
Caesalpinioideae	*Intsia palembanica* Miq	leaf	0	c
Caesalpinioideae	*Peltophorum dasyrachis* Kurz	leaf	0	c
Caesalpinioideae	*Peltophorum pterocarpum* Back. ec Heyne	leaf	0	e
Caesalpinioideae	*Pterobium microphyllum* Miq	leaf	0	c
Caesalpinioideae	*Saraca declinata* Miq	leaf	0	c
Caesalpinioideae	*Saraca indica* L.	leaf	0	d
Caesalpinioideae	*Saraca pierreana* Craib	leaf	0	c
Caesalpinioideae	*Saraca thaipingensis* Contly ex. Prain	leaf	0	c
Caesalpinioideae	*Senna alata* (L.) Roxb.	leaf/seed	0	c
Caesalpinioideae	*Senna garrettiana* (Craib) Irwin et Barneby	leaf	0	c

Table 4. (Continued)

Family	Plant species	Part	HCN content (ppm)	Source
Caesalpinioideae	*Senna hirsute* (L.) Irwin&Barneby	leaf/seed	0	c
Caesalpinioideae	*Sindora echinocalyx* Prain	leaf	0	c
Caesalpiniodeae	*Sindora siamensis*	leaf	0	d
Caesalpiniodeae	*Tamarindus indica* L.	leaf	0	e
Capparaceae	*Capparis micracantha* DC.	leaf	0	c
Capparaceae	*Capparis tenera* Dalz.	leaf	0	e
Capparaceae	*Cleome gynandra* L.	leaf	0	b
Capparaceae	*Cleome viscose* L.	leaf	0	b
Capparaceae	*Cratera adansonii* DC. subsp. Trifoliata (Roxb.) Jacobs.	leaf	0	c
Capparaceae	*Crateva magna* (Lour.) DC.	leaf	0	c
Caricaceae	*Carica papaya* L.	leaf	0	c
Compositae	*Camchaya spinulifera*	leaf	0	d
Compositae	*Elephantopus scaber* L.	leaf	0	d
Dioscoreaceae	*Dioscorea hispida* Dennst. var. hispida	leaf	0	b
Euphorbiaceae	*Acalypha hispida* Burm. f.	leaf/flower	0	c
Euphorbiaceae	*Acalypha indica* Linn.	leaf	1,020	b
Euphorbiaceae	*Antidesma Leucocladon* Hook. f.	leaf	0	d
Euphorbiaceae	*Antidesma sootepense* Craib.	leaf	0	e
Euphorbiaceae	*Aporosa aurea* Hook. f.	leaf/seed	0	c
Euphorbiaceae	*Aporosa prainian* a King	leaf	0	d
Euphorbiaceae	*Aporusa wallichii* Hk. f.	leaf	0	e
Euphorbiaceae	*Baccaurea ramiflora* Lour.	leaf	0	c
Euphorbiaceae	*Balakata baccata* Roxb.	leaf	0	e
Euphorbiaceae	*Bridelia affinis* Craib	young leaf/leaf	0	e
Euphorbiaceae	*Bridelia ovata* Decne	leaf	0	c
Euphorbiaceae	*Chaetocarpus castanocarpus* (Roxb.) Thwaites	leaf	0	d
Euphorbiaceae	*Cleidion speciflorum* Merr.	young leaf/leaf	0	e
Euphorbiaceae	*Cleistanthus helferi* Hook. f.	leaf	0	d
Euphorbiaceae	*Croton longissimus* Ariy Shaw	leaf	0	c
Euphorbiaceae	*Croton oblongifolius* Roxb.	leaf	0	c

Table 4. (Continued)

Family	Plant species	Part	HCN content (ppm)	Source
Euphorbiaceae	*Croton tiglium* L.	leaf	0	c
Euphorbiaceae	*Elateriospermum tapos* Blume	leaf	7	c
Euphorbiaceae	*Euphorbia antiquorum* Linn.	branch	0	c
Euphorbiaceae	*Euphorbia lacei* Craib.	branch	0	c
Euphorbiaceae	*Euphorbia tirucalli* L.	branch	0	c
Euphorbiaceae	*Euphorbia thymifolia* L.	leaf	0	c
Euphorbiaceae	*Excoecaria cochinchinensis* Lour.	leaf	0	c, e
Euphorbiaceae	*Glochidion perakense* Hook. f.	young leaf/leaf	0	a
Euphorbiaceae	*Glochidion sphaerogynum* (Mull. Arg.) Kurz.	leaf	0	e
Euphorbiaceae	*Hevea brasiliensis*	leaf	2,961	d
Euphorbiaceae	*Hura crepitans* Linn.	leaf	0	e
Euphorbiaceae	*Jatropha cureas* L.	leaf	0	c
Euphorbiaceae	*Jatropha podagrica* Hook. f.	leaf	0	c
Euphorbiaceae	*Jatropha multifida* L.	leaf/seed	0	c
Euphorbiaceae	*Manihot esculenta* Crantz	leaf	1,013	b
Euphorbiaceae	*Macaranga denticulate Muell. Arg.*	leaf	0	e
Euphorbiaceae	*Mallotus paniculatus* Muell. Arg	leaf	0	e
Euphorbiaceae	*Phyllanthus amarus* Schum & Thonn.	leaf	0	e
Euphorbiaceae	*Phyllanthus emblica* Linn.	leaf/seed	0	c
Euphorbiaceae	*Phyllanthus geoffrayi* Beille	leaf	0	a
Euphorbiaceae	*Phyllanthus pulcher* Wall. ex Mull.Arg.	leaf	0	c
Euphorbiaceae	*Phyllanthus sp.*	leaf	0	e
Euphorbiaceae	*Suregada multiflorum* Baill	leaf/seed	0	c
Euphorbiaceae	*Trewia nudiflora* L.	leaf	0	e
Flacourtiaceae	*Casearia grewiaefolia Vent.*	leaf	0	e
Flacourtiaceae	*Homalium ceylanicum* (Gardn.) Benth.	leaf	0	e
Flacourtiaceae	*Hydnocarpus ilicifolia* King	leaf	707	b
Gramineae	*Bambusa bambos* (L.) Voss	young shoot	1,822	a
Gramineae	*Zea mays*	seed	0	a
Laeaceae	*Aquilaria malacensis* Lamk. Thyme	leaf	0	c
Lauraceae	*Litsea elliptica* Blume	leaf	0	c
Menispermaceae	*Stenotaphrum pierrei* Diels	leaf	0	c

Table 4. (Continued)

Family	Plant species	Part	HCN content (ppm)	Source
Mimosoideae	*Acacia catechu* (L.f.) Willd.	leaf	0	c
Mimosoideae	*Acacia catechu* (L.f.) Willd	leaf/seed	0	c
Mimosoideae	*Acacia comosa* Gagnep.	young leaf/leaf	0	e
Mimosoideae	*Acacia farnesiana* (L.) Willd.	leaf	4	c
Mimosoideae	*Acacia mangium* Willd.	leaf	0	d
Mimosoideae	*Acacia pennata* (L.) Willd. subsp. Insuavis (Lace) I.C.Nielsen	young leaf/leaf	0	a
Mimosoideae	*Adenanthera pavonina* L.	leaf	0	d
Mimosoideae	*Albizia myriophylla* Bonth	leaf	0	c
Mimosoideae	*Albizia odoratissima* (L.f.) Benth	leaf	0	c
Mimosoideae	*Albizia procera* (Roxb.) Benth	leaf	0	d
Mimosoideae	*Archidendron jiringa* (Jack) I.C.Nielsen	leaf/seed/seedling	0/ 0/ 17	a
Mimosoideae	*Archidendron bubalinum* (Jack) I.C.Nielson	young seed	0	a
Mimosoideae	*Calliandra haematocephala* Hassk.	leaf	0	e
Mimosoideae	*Calliandra* sp.	leaf	0	e
Mimosoideae	*Entada pursaetha* DC.	leaf	0	c
Mimosoideae	*Leucaena leucocephala* (Lam.) de Wit	leaf/seed	0	a
Mimosoideae	*Leucaena leucocephala* (Lam.) de Wit	leaf	0	c
Mimosoideae	*Parkia speciosa* Hassk.	leaf	0	c
Mimosoideae	*Xylia sylocarpa* (Roxb.) Taub. var. kerrii (Craib&Hutch) I.C. Nielson	leaf	0	d
Myrtaceae	*Eugenia paniala* Roxb.	young leaf/leaf	0	e
Myrtaceae	*Syzygium cumini* (L.) Skeels	leaf	0	e
Papilionaceae	*Abrus precatorius* Linn.	young leaf/leaf	0	e
Papilionoideae	*Arachis hypogaea*	seed	0	a
Papilionoideae	*Crotalaria spectabilis* Roth subsp-parvibracteata	leaf	0	c
Papilionoideae	*Dalbergia abbreviate* Craib	leaf	0	c
Papilionoideae	*Dalbergia cochinchinensis*	leaf	1,795	d
Papilionoideae	*Dalbergia cultrate* Benth.	young leaf/leaf	0	e
Papilionoideae	*Dalbergia floribunda* Roxb.	leaf	655	e

Table 4. (Continued)

Family	Plant species	Part	HCN content (ppm)	Source
Papilionoideae	*Dalbergia oliceri* Gamble	young leaf/leaf	0	e
Papilionoideae	*Desmodium renifolium* Schindl. var. Oblatum.	leaf	0	d
Papilionoideae	*Erythrina variegate* Linn.	leaf	0	c
Papilionoideae	*Flemingi sootepensis* Craib	leaf	0	e
Papilionoideae	*Macroptilium lathyroides* (L.) Urb.	leaf	0	c
Papilionoideae	*Millettia artopurpurea* Beatl.	leaf	0	c
Papilionoideae	*Millettia kangensis* Craib	leaf	0	e
Papilionoideae	*Mucuna pruriens (L.) DC. var. pruriens*	leaf	0	e
Papilionoideae	*Ormosia sumatrana* (Miq.) Prain	leaf	0	c
Papilionoideae	*Pterocarpus indicus* Willd.	leaf	0	d
Papilionoideae	*Tecoma stans* (L.) Kunth	leaf	0	d
Passifloraceae	*Passiflora cocinea* Aubl.	young leaf/leaf	833 /0	e
Passifloraceae	*Passiflora edulis*	leaf	1,085	e
Passifloraceae	*Passiflora quadrangularis* L.	leaf	1,169	e
Proteaceae	*Heliciopsis terminalis* (Kurz) Sleumer	leaf	0	d
Rosaceae	*Eriobotrya bengalensis*	leaf	0	e
Rosaceae	*Prunus cerasoides* D. Don.	young leaf/leaf	0	e
Rosaceae	*Prunus mume* Sieb.	leaf	0	e
Rosaceae	*Rhapiolepis indica* Lindl. Ex Kerr.	leaf	0	e
Rosaceae	*Rubus alceifolius* Poir.	young leaf/leaf	0	e
Rosaceae	*Rubus ellipticus* J. E. Smith	leaf	0	e
Sapindaceae	*Litchi chinensis* Sonn.	seed	0	a

* Source – (a) Fresh market in Hat Yai, Songkhla, Thailand; (b) Peninsular Botanic garden, Thung Khai, Trang, Thailand; (c) Prince of Songkla University, Hat Yai, Songkhla, Thailand; (d) Southern Flora with Its Thai Literature Collection, Songkhla, Thailand; (e) Queen Sirikit Botanic Garden, Chiangmai, Thailand.

The positive cyanide test has been observed in two species of *Dalbergia* which belong to Papilionoideae. The detected cyanide contents were 1,795 and 655 ppm in mature leaves of *D. cochinchinensis* and *D. floribunda* Roxb, respectively. However, no cyanide content was observed in both mature and young leaves of *Dalbergia cultrate* Benth. No cyanide liberation was detected in another 16 species in this family.

The presence of strong cyanogenesis in all 3 selected species of Passifloraceae were investigated in leaves of *Passiflora quadrangularis*, *P. edulis* and young leaves of *P. coccinea* with the cyanide contents of 1,169, 1,085 and 833 ppm, respectively. However, no cyanide liberation was found in mature leaves of *P. coccinea*. The previous studies reported that the cyanide content in Passifloraceae was ranged from 1.5–3,340 ppm (Adsersen *et al.*, 1993; Bylov *et al.*, 2004; Chassagne and Crouzet, 1998).

2.4.2 Effect of pH and temperature on stability of mandelonitrile

Since cyanohydrins are the terminal intermediate compounds in the decomposition pathway of cyanogenesis, it is necessary to determine the stability of these compounds in several conditions of pH and temperature. In this study, racemic mandelonitrile was used as cyanohydrin.

The effect of pH on stability of mandelonitrile was shown in Figure 26. The decomposition rate of mandelonitrile increased when increase pH. The stability of mandelonitrile was observed in acidic pH between 3 and 4, but pH more than 4.5 caused rapidly decomposition of the compound.

Temperature affected on the decomposition of mandelonitrile as shown in Figure 27. Increase of temperature accelerated the decomposition of mandelonitrile, while the mandelonitrile was stable at low temperature (10 °C).

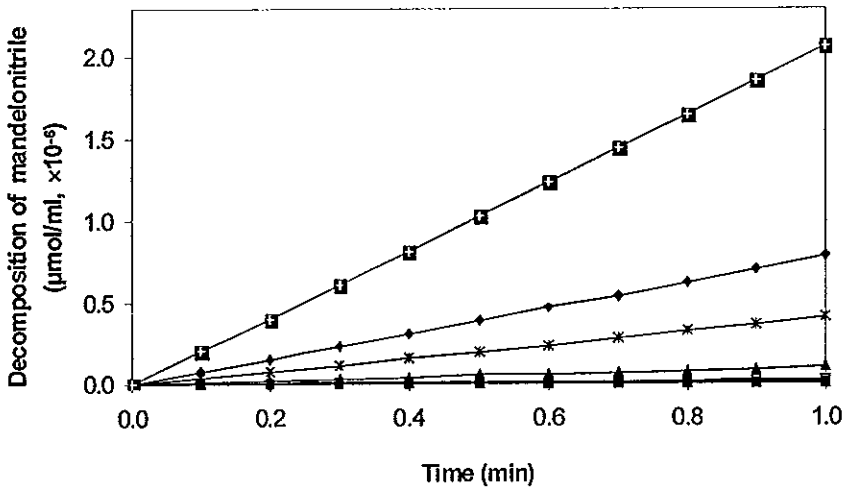


Figure 26. Effect of pH on stability of mandelonitrile. The decomposition of mandelonitrile was performed at 30 °C in 50 mM citrate phosphate buffer at various pH. (■) pH 3; (□) pH 4.0; (▲) pH 4.5; (X) pH 5.0; (♦) pH 5.5; (▣) pH 6.0.

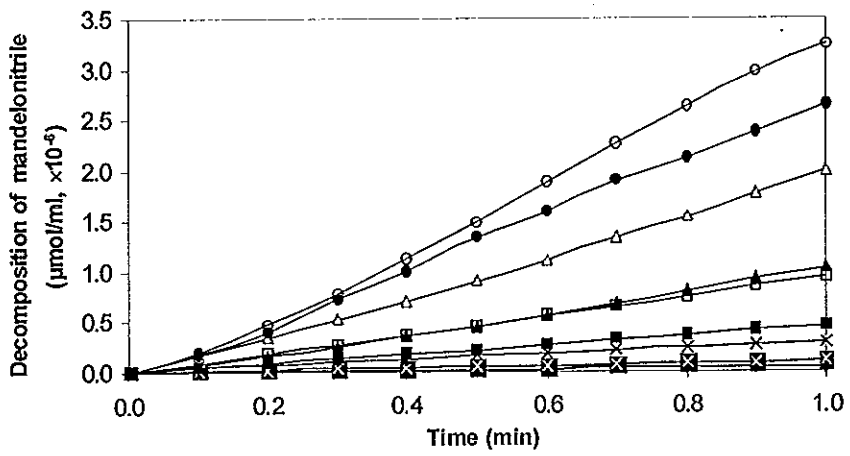


Figure 27. Effect of temperature on stability of mandelonitrile. The decomposition of mandelonitrile was performed in 50 mM citrate phosphate buffer, pH 5.5 at various temperature. (▣) 10 °C; (X) 20 °C; (■) 30 °C; (□) 35 °C; (▲) 40 °C; (Δ) 45 °C; (●) 50 °C; (○) 60 °C.

2.4.3 Screening of Hydroxynitrile lyase (HNL) activity

The activity of HNL in crude extracts of cyanogenic plant was determined. Leaves of *Hevea brasiliensis*, leaves of *Manihot esculenta*, and leaves and seeds of *Passiflora edulis*, were already known as sources of HNL (Asano *et al.*, 2005). *H. brasiliensis* and *M. esculenta* leaves extracts were used as positive control for (*S*)-HNLs, while leaves extract of *P. edulis* was used as positive control for (*R*)-HNLs (Asano *et al.*, 2005).

The results on screening of HNL activity was shown in Table 5, crude plant extracts from leaves of *Lisia spinosa, Xantosoma nigrum, Afzelia xylocarpus, Acalypha indica, Elateriospermum tapos, Hydnocarpus ilicifolia, Dalbergia cochinchinensis, Dalbergia ovata, Passiflora cocenea, Passiflora quadrangularis, Saraca declinata, Saraca pierreana, Acacia farnesiana, Heliciopsis terminalis*, shoot of *Bambusa bambos*, seeds of *Annona squmosa*, and seedling of *Archidendron jiringa* was inactive.

The HNL activity was observed in crude extract of leaves (28.8 U/g), seeds (19.6 U/g) and rind (14.5 U/g) of *Passiflora edulis* (passion fruit) with specific activity of 1.47, 1.23 and 0.92 U/mg, respectively. The HNL extracted from *P. edulis* (*Pe*HNL) performed stereoselectivity on the synthesis of (*R*)-mandelonitrile indicating the enzyme was (*R*)-HNL. The enantiomeric excess (*e.e*) of (*R*)-mandelonitrile synthesized by *Pe*HNL was demonstrated in Table 6. *Pe*HNL extracted from leaves, seeds, and rind showed ability in cyanohydrin synthesis in buffer system with *e.e.* of 54.6, 37.9, and 33.2 %, respectively.

Table 5. Hydroxynitrile lyase activity of some cyanogenic plants.

Family	Plant	Part	Configuration	Activity (U/g sample)	Specific activity[a] (U/mg)
Araceae	*Lisia spinosa*	leaf	-	-	0
Araceae	*Xantosoma nigrum*	leaf	-	-	0
Caesalpinioideae	*Afzelia xylocarpus*	leaf	-	-	0
Euphorbiaceae	*Acalypha indica*	leaf	-	-	0
Euphorbiaceae	*Elateriospermum tapos*	leaf	-	-	0
Euphorbiaceae	*Hevea brasiliensis*[b]	leaf	*S*	33.2	3.28
Euphorbiaceae	*Manihot esculenta*[b]	leaf	*S*	14.5	2.13
Flacourtiaceae	*Hydnocarpus ilicifolia*	leaf	-	-	0
Papilionoideae	*Dalbergia cochinchinensis*	leaf	-	-	0
Papilionoideae	*Dalbergia ovata*	leaf	-	-	0
Passifloraceae	*Passiflora cocenea*	leaf	-	-	0
Passifloraceae	*Passiflora edulis*[b]	leaf	*R*	28.8	1.47
Passifloraceae	*Passiflora edulis*[b]	Seed	*R*	19.6	1.23
Passifloraceae	*Passiflora edulis*	Rind	*R*	14.5	0.92
Passifloraceae	*Passiflora quadrangularis*	Leaf	-	-	0
Caesalpinioideae	*Saraca declinata*	leaf	-	-	0
Caesalpinioideae	*Saraca pierreana*	leaf	-	-	0
Mimosoideae	*Acacia farnesiana*	leaf	-	-	0
Gramineae	*Bambusa bambos*	shoot	-	-	0
Annonaceae	*Annona squamosa*	seed	-	-	0
Mimosoideae	*Archidendron jiringa*	seedling	-	-	0
Proteaceae	*Heliciopsis terminalis*	leaf	-	-	0

[a] Determined the production of mandelonitrile using HPLC with CHIRALCEL OJ-H column and employed benzaldehyde and KCN as substrate.

[b] Known sources of HNLs.

Table 6. The ability in cyanohydrin synthesis of crude HNL from various parts of *P. edulis*.

Plant species	Part	Mandelonitrile	
		e.e. (%)	Configuration
P. edulis	Leaf	54.6	*R*
	Seed	47.9	*R*
	Rind	33.2	*R*

2.5 Discussion

The number of cyanogenic plants was estimated at least 2,650 species, while HNLs were discovered in only 26 plant species (Asano *et al.*, 2005). HNLs established themselves to be a valuable enzyme in asymmetric synthesis of cyanohydrins which are versatile building blocks to produce the commercial products in fine chemicals, pharmaceuticals, and agro-chemicals industries. Therefore, screening for cyanogenic plants and novel HNLs should be carried on, and the relationship between the occurrence of cyanogenesis in plants and the presence of HNLs is of interest.

The occurrence of cyanogenesis in 176 plant species from 26 families which cyanogenesis phenomena has been reported was screened for the presence of HCN, and 16 plant species were cyanogenic plant releasing HCN. Among these plants, 5 novel sources of cyanogenic plants were discovered which are *Afzelia xylocarpus, Archidendron jiringa, Dalbergia floribunda, Xantosoma nigrum, Hydnocarpus ilicifolia.* Since HCN content below 1.0-1.8 ppm was undetectable by picrate method (Seigler *et al.*, 1979), some plant samples producing very little quantity of HCN might not give positive test in this study. The difference in HCN content is caused by two main reasons. The first reason is an ability of plant to synthesize and accumulate cyanogenic glycosides. The other is the presence of degrading enzymes, β-glucosidase, in the same plant (Selmar *et al.*, 1991; Conn, 1980b). Only the plant containing both cyanogenic glycosides and the hydrolysis enzyme could release HCN. Moreover, cyanogenesis might be affected by ecological factors, cultivar-dependence, plant age, growth phases and the

part of plant used for the test (Janzen *et al.*, 1980; Selmar *et al.*, 1987; Selmar *et al.*, 1991; Lieberei *et al.*, 1986).

HNLs, one of the enzymes involve in the cyanogenesis of higher plants, catalyze the decomposition of cyanohydrins into HCN and corresponding carbonyl compounds. HNLs are classified according to their steriospecificity into (*R*)- and (*S*)-HNLs which catalyze the decomposition or synthesis of (*R*)- and (*S*)-cyanohydrins, respectively.

On this present study, the (*R*)-HNL from *P. edulis* (passion fruit) was discovered in several parts including leaves, seeds and rind. Rind of *P. edulis* is an interesting source of (*R*)-HNL, since 60% of passion fruit is rind and it is discarded after extraction of juice generates a major waste problem for industries and environment (Rodriguez-Amaya, 2003). The *e.e.* on (*R*)-mandelonitrile synthesis by (*R*)-*Pe*HNL in the system of citrate phosphate buffer as shown in Table 3 could be further improved by optimization of reaction or employing the enzyme in organic medium.

From the results, HNLs were observed in some cyanogenic plants, but not in all of cyanogenic plants. Cyanogenic plants can spontaneously release HCN without the presence of HNLs, because the intermediate cyanohydrins are unstable and easily decompose to HCN and corresponding carbonyl compounds. The intemidiate cyanohydrins were unstable under the neutral to alkaline pH and high temperature.

The presence of HNLs in plant might be controlled by genetic control. Some cyanogenic plants were reported to be sources of HNLs such as *Manihot esculenta*, *Hevea brasiliensis*, *Sorghum bicolor*, and *Prunus amygdalus*, and the nucleotide sequences encoding to the HNLs in these cyanogenic plants have been studied and reported elsewhere (Hickel *et al.*, 1996; Sharma *et al.*, 2005). In contrary, (*R*)-HNL was also observed from the non-cyanogenic plant *Arabidopsis thaliana* (mouse-ear cress). The nucleotide sequence of *A. thaliana* showed similarity to that of HNL from *M. esculenta* and *H. brasiliensis*, and the target gene encoding to HNL from *A. thaliana* was cloned and expressed in *Escherichia coli* successfully (Andexer *et al.*, 2007).

2.6 Conclusion

Several cyanogenic plants were investigated by screening for the presence of cyanide by picrate paper method. In the same plant family, some plants were cyanogenic plants, while some plants were not. The releasing of cyanides might be influenced by the presence of both hydrolytic enzyme (β-glucosidase) and cyanogenic glycoside collected in plant tissue. The cyanogenic glycoside might be hydrolyzed by β-glucosidase yielding the intermediate cyanohydrins, and then the cyanohydrins might be decomposed spontaneously or catalyzed by HNLs to cyanide and carbonyl compounds. Actually, the cyanohydrin is unstable in neutral to alkaline pH and ambient to high temperature, then the releasing of cyanide from cyanohydrin might occurred spontaneously without HNLs. The presence of HNLs was not observed in every cyanogenic plants, since the presence of HNL might be controlled genetically by the plant genome. The novel HNL from *Passiflora edulis* was observed in leaves, seeds, and rind of the plant and the enzyme showed promising ability in cyanohydrin synthesis.

CHAPTER 3

A novel hydroxynitrile lyase from *Passiflora edulis* catalyze the asymmetric synthesis of (*R*)-mandelonitrile

3.1 Abstract

Asymmetric synthesis of (*R*)-mandelonitrile in biphasic system employing a hydroxynitrile lyase from *Passiflora edulis* (*Pe*HNL) was reported for the first time. Several parameters influenced the enantiomeric purity of product and initial velocity of the reaction. Both pH and temperature were important parameters to control the enantiomeric purity of the product. The optimum pH and temperature were pH 4 and 10 °C, respectively. At the optimum conditions, the spontaneous non-enzymatic reaction yielding the racemic of mandelonitrile was almost suppressed. The *Pe*HNL performed more than 80% residual activity in the system of methyl-t-butyl ether, dibutyl ether (DBE), hexane, and diisopropyl ether while diethyl ether and ethyl acetate were not suitable solvents. The initial velocity was markedly affected by type of organic solvents in biphasic system. When using the organic solvent with log *P* lower than 3.5, high enantiomeric purity was obtained. The highest initial velocity of reaction and enantiomeric purity of (*R*)-mandelonitrile were obtained in biphasic system of DBE with the aqueous phase content of 30% (v/v). The optimum substrate concentrations were 250 mM for benzaldehyde and 900 mM for acetone cyanohydrin, while the optimum enzyme concentration was 26.7 units/ml. The high enantiomeric purity of (*R*)-mandelonitrile was successfully obtained with 31.6% conversion and enantiomeric excess of 98.6%. The enzyme performed the considerably reusability in fourth batch reaction with high enantiomeric purity of product.

3.2 Introduction

Since the demand in chiral intermediate compounds with high enantiomeric purity has been increased markedly in many industries such as foods, chemicals, pharmaceuticals, and agrochemicals, the synthesis and production of chiral compounds have been of interest to support these demands (Pollard and Woodley, 2006). Chiral cyanohydrins are important synthetic intermediates for many industrial products due to both functional groups of cyanohydrins, the hydroxyl and cyanide moiety attached to the same carbon, can be easily converted into a wide range of other chiral products such as α-hydroxy aldehyde and ketones, β-amino alcohols, α-fluoro cyanides, etc (North, 2003; North, 2004). One possibility for synthesis of chiral cyanohydrins is the enantioselective addition of HCN to a prochiral aldehyde or ketone with hydroxynitrile lyases (HNLs) as biocatalysts (Hickel *et al.,* 1996a; Griengl *et al.,* 1997; Nanda *et al.,* 2005; Liu *et al.,* 2008).

The enantiomeric purity of cyanohydrins obtained from the single aqueous phase reaction catalyzed by HNLs was disturbed by non-enzymatic reaction which formed the undesired racemic of the products. The biphasic system of buffer and immiscible organic solvent was introduced to solve this problem and provides many advantages such as high efficiency, cost effective, and easy down stream processing because product is extracted into organic phase while the enzyme is remaining in the aqueous phase (Sharma *et al.,* 2005). However, several parameters are involved in a complex interaction in the biphasic reaction of HNLs.

The difference in some parameters influencing on the initial velocity of reaction and enantiomeric purity of the product in biphasic reaction have been observed and reported in several enantioselective enzyme, the parameters are, for example, pH, temperature, organic solvent, and source of enzyme, etc (Watanabe *et al.,* 2004; Castro and Knubovets, 2003; Wehtje *et al.,* 1997). The previous researches on the syntheses of chiral cyanohydrins in biphasic system were studied on the HNLs from *Manihot esculenta* (*Me*HNL), *Hevea brasiliensis* (*Hb*HNL), *Sorghum bicolor* (*Sb*HNL), and *Prunus amygdalus* (*Pa*HNL) (Gerrits *et al.,* 2001; Bauer *et al.,* 1999; Costes *et al.,* 1999; Loos *et al.,* 1995; Hickel *et al.,* 2001; Presson *et al.,* 2002, Cascao-Pereira *et al.,* 2003, Lin *et al.,* 1999).

Passiflora edulis, commonly known as passion fruit, was reported to be a novel source of (*R*)-HNL by our group (Asano *et al.*, 2005). The HNL from *P. edulis* (*Pe*HNL) performed the ability in the synthesis of (*R*)-mandelonitrile and (*R*)-methylpropylketone cyanohydrin from benzaldehyde and 2-pentanone, respectively, in buffer system employing potassium cyanide as a cyanide source (Asano *et al.*, 2005). There is no data available for the cyanohydrin synthesis by *Pe*HNL in two-phase system with high enantiomeric purity of product.

P. edulis is one of important commercial fruits and cultivated on a large scale in Thailand, Brazil, Kenya, New Zealand, etc. The fruits are used in the industrial production of concentrated juice, jam, nectar, confectionary, etc. In the extraction of juice from the passion fruit, about two-thirds of the bulk is discarded, of which 90% is rind and 10% is seeds (Rodriguez-Amaya, 2003). The passion fruit rind, by-product resulting from the processing, generates a major waste problem for the industries and environment.

In this study, the rind of passion fruit was used as the *Pe*HNL source. We aimed to study on the characteristics of *Pe*HNL in biphasic system and synthesize (*R*)-mandelonitrile to achieve the highest enantiomeric purity of the product and the highest initial velocity of the reaction for the further industrial application of the *Pe*HNL. Furthermore, transcyanation reaction (Figure 28) employing acetone cyanohydrin as a cyanide source was carried out along the experiment to avoid the direct handling of hazardous hydrocynic acid (HCN).

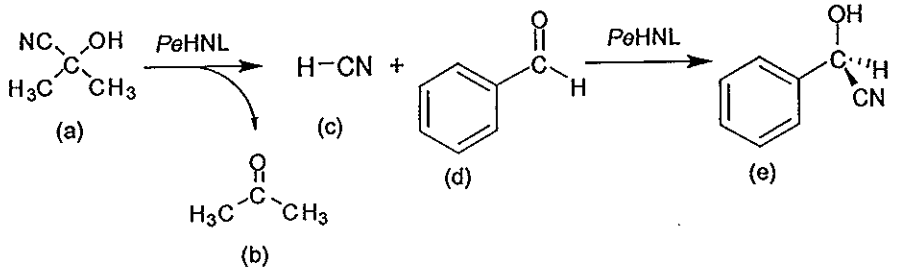


Figure 28. The synthesis of (*R*)-mandelonitrile by transcyanation of acetone cyanohydrin and benzaldehyde using *Pe*HNL. Acetone cyanohydrin (a) was cleaved and released acetone (b) and hydrocyanic acid (c) by the enzymatic reaction of *Pe*HNL. Then, the *Pe*HNL catalyze the asymmetric addition of hydrocyanic acid to benzaldehyde (d) yielding the (*R*)-mandelonitrile (e).

3.3 Materials and Methods

3.3.1 Materials

Passion fruits (*Passiflora edulis*) were purchased from fresh market in Songkhla and Chumporn (Thailand) and stored at -20 °C. Benzaldehyde (redistilled, 99.5+%; Sigma-Aldrich, Inc., USA) and acetone cyanohydrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and all of chemicals used in the experiments were purchased from commercial sources and used without further purification. (*R*)-(+)-Mandelonitrile (Sigma-Aldrich, Inc., USA) was used as standard in chiral HPLC analysis. Chiral HPLC analysis was performed using chiralcel OJ-H column (Diacel Chemical Industries Ltd., Osaka, Japan) with SPD-10A VP UV-vis detector (Shimadzu, Kyoto, Japan) at 254 nm. Eluting solvent was 85% hexane and 15% isopropanol by volume (Ueatrongchit *et al.*, 2008).

3.3.2 Crude enzyme preparation

Rind of passion fruits was cut into small pieces, frozen by liquid nitrogen and ground in homogenizer. The powder obtained was extracted in 10 mM potassium phosphate buffer, pH 6.0 with 3% polyvinylpyrolidone. The extract was filtered and squeezed through 4 layers of cheesecloth. Pectinase (10 U/100 ml) was added to the filtrate and stirred for 6 h to hydrolyze pectin. The precipitate occurred was discarded after centrifuged at 28,000 *g* for 30 min at 4 °C. The enzyme was separated from the supernatant by 30-80% $(NH_4)_2SO_4$ precipitation, dissolved and dialyzed against the 10 mM potassium phosphate buffer, pH 6.0. The precipitate during the dialysis was discarded by centrifugation at 28,000 *g* for 30 min at 4 °C, the supernatant was lyophilized and used as crude enzyme.

3.3.3 Activity assay

Reaction mixture in total volume of 1.0 ml was prepared in a micro-tube, 1.0 M of benzaldehyde (in DMSO, 40 µl) was added to sodium citrate buffer (400 mM, pH 4.0),

followed by addition of enzyme solution and KCN solution (1.0 M, 100 µl). The reaction was monitored by taking small aliquot of reaction mixture (100 µl) and extracting with 700 µl of organic solvent (85% n-hexane, 15% isopropanol by volume). The organic layer containing benzaldehyde, (*R*)- and (*S*)-mandelonitrile obtained after centrifugation at 15,000 g at 4 °C for 1 min was analyzed by chiral HPLC. The retention time of benzaldehyde, (*R*)- and (*S*)-mandelonitrile were 4.9, 10.2, and 12.7 min, respectively. The enzyme activity was calculated from progress linear curve of reaction in the first five minutes.

One unit of HNL activity was defined as the amount of enzyme that produced 1 µmol of optically active mandelonitrile from benzaldehyde per min under the assay condition (Asano *et al.*, 2005).

3.3.4 Transcyanation reaction in biphasic system

The reaction (total volume of 1.5 ml) was performed in 2.0 ml micro-tube. The organic solvent containing benzaldehyde was mixed with citrate-phosphate buffer (400 mM) containing crude enzyme powder. The reaction was initiated by the addition of acetone cyanohydrin, the mixture was shaken at 1500 rpm in incubator shaker (BioShaker M-BR-022UP). Aliquot of sample (50 µl) was withdrawn from the organic phase at different interval times, mixed with HPLC solvent (100 µl; n-hexane:2-propanol, 85:15). The initial velocity, conversion and enantiomeric excess (*e.e.*) were analyzed by chiral HPLC. The initial velocity and conversion was analyzed by using standard curve of (*R*)-mandelonitrile, and the *e.e.* was determined by calculation the peak areas of the two enantiomers using Eq. (1):

$$\text{Enantiomeric excess, } e.e.\ (\%) = [R\text{-}S]/[R+S] \times 100 \qquad (1)$$

where R and S represent the concentrations of the (*R*)-mandelonitrile and (*S*)-mandelonitrile, respectively.

Details of the pH, temperature, benzaldehyde and acetone cyanohydrin concentration, organic solvent, volume of the aqueous buffer and enzyme concentration were specific for each case.

3.3.5 Influence of organic solvents on enzyme stability

Different organic solvents were mixed with 400 mM citrate-phosphate buffer, pH 4.0 (ratio 1:1 by volume) and equilibrated under shaking for 60 min. Enzyme solution (50 µl, 5 units) was injected into the aqueous phase and gently mixed not to disturb the interface. Then, the enzyme activity at zero time was determined under the saturated solvent in aqueous phase. The mixtures were shaken to obtain the good mixing between two phases at 1,500 rpm and incubated for 12 h. Then, the aqueous phase was withdrawn to assay the remaining activity of the enzyme.

3.3.6 Reusability

Reusability of *Pe*HNL in batch transcyanation reaction of benzaldehyde (250 mM) and acetone cyanohydrin (900 mM) in biphasic system of 400 mM citrate-phosphate buffer, pH 4.0 (5 ml) and dibutyl ether (5 ml) was initiated by addition of the *Pe*HNL powder (26.7 units) shaking at 1500 rpm at 10 °C for 3 h. Then the aqueous phase containing the enzyme was recovered and dialyzed against the 400 mM citrate-phosphate buffer, pH 4.0 and was reused for the next batch reaction under the same conditions.

3.4 Results

3.4.1 Effect of buffer pH

Effect of pH was monitored in the biphasic system of buffer and DIPE. The enantioselectivity and initial activity of the *Pe*HNL were influenced by pH of reaction buffer. The optimum pH of *Pe*HNL was pH 6.0. The activity was increased when increased pH from 3.0-6.0. It was decreased when increased pH more than the optimum (Figure 29a).

In contrast to the initial velocity of the enzyme, the *e.e.* of 99% for (*R*)-mandelonitrile was obtained at pH 3.0-4.0, but it was markedly decreased at pH more than 4.0 due to the rapidly increasing of non-enzymatic reaction yielding the racemic of both enantiomers.

It was not suitable to perform reaction at the optimum pH since the low enantiomeric purity (51% *e.e.*) was obtained. The alkaline pH accelerated the release of HCN from acetone cyanohydrin (Fomunyan *et al.,* 1985) leading to the increase of non-enzymatic reaction. To obtain the high *e.e.* of 99%, pH 4.0 was chosen for transcyanation of (*R*)-mandelonitrile along the experiment.

3.4.2 Effect of reaction temperature

Effect of temperature on the *Pe*HNL in transcyanation of (*R*)-mandelonitrile was examined in the range of 4-50 °C. As shown in Figure 29b, the optimum temperature of *Pe*HNL was 40 °C. The initial velocity of reaction increased when temperature was increased from 4-40 °C, followed by a decrease at higher temperature. The elevation of temperature might increase the chance of collision between enzyme and substrate molecules to form enzyme-substrate complexes, therefore the initial velocity of reaction was improved. In contrast to the initial velocity, the increase of *e.e.* was observed with a decrease in the temperature. The *e.e.* of 99% was obtained at low temperature between 4-10 °C.

Increasing of temperature accelerated the non-enzymatic reaction, therefore the low *e.e.* of product was found. Since the greatest *e.e.* and the activity around 50% of the highest activity were found at 10 °C, thereby 10 °C was selected as the optimum temperature for this reaction to avoid the non-enzymatic reaction.

3.4.3 Organic solvents

Effect of organic solvents on transcyanation of (*R*)-mandelonitrile synthesis by *Pe*HNL was investigated in the biphasic system of buffer and various organic solvents. The organic solvents used in this study were ethyl acetate (EA; log *P* 0.67), diethyl ether (DEE; log *P* 0.85), methyl-t-butyl ether (MTBE; log *P* 1.4), diisopropyl ether (DIPE; log *P* 1.9), dibutyl ether (DBE; log *P* 2.9) and hexane (HEX; log *P* 3.5).

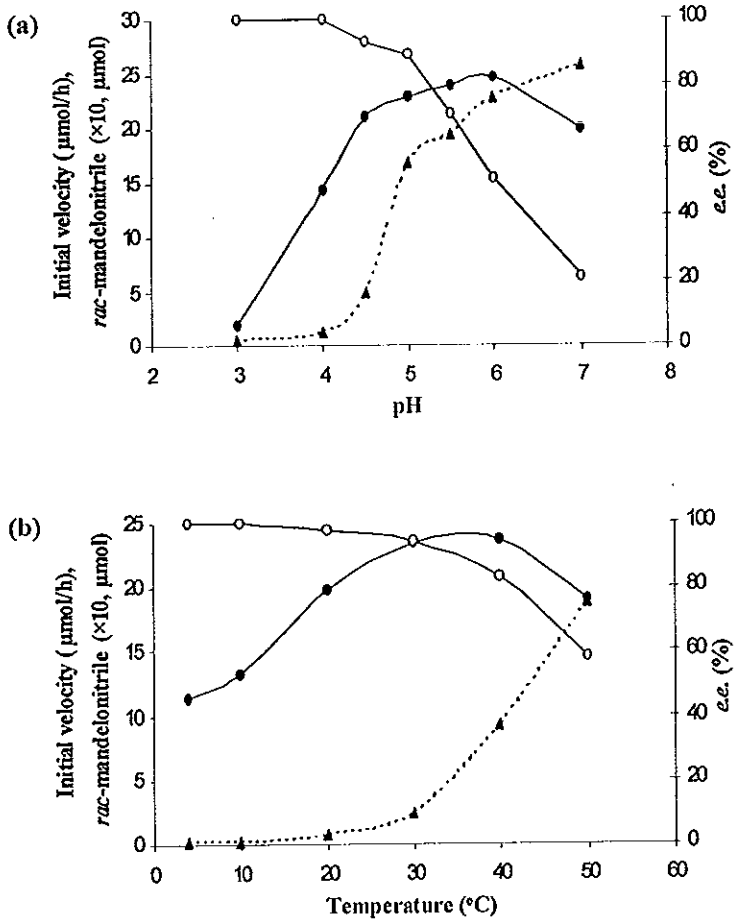


Figure 29. Effect of pH (a) and temperature (b) on transcyanation reaction catalyzed by *Pe*HNL. Initial velocity (●), enantiomeric excess (*e.e.*) (O), and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer and DIPE, containing 5 U of *Pe*HNL. The reaction was performed at 30 °C for pH effect and pH 4.0 for temperature effect.

The initial velocity of *Pe*HNL in biphasic systems did not correlate with log *P* of organic solvents (Figure 30a). The highest initial velocity was achieved in biphasic system of DBE (16.8 µmol/h) while the enzyme in DIPE performed moderate initial velocity (9.6 µmol/h). EA, DEE, MTBE, and HEX were not suitable for (*R*)-mandelonitrile synthesis by *Pe*HNL due to the inactivation effect of organic solvent to the enzyme.

The stability of *Pe*HNL was monitored after incubation with the organic solvents for 12 h, the residual activity of the enzyme was compared with the initial activity in aqueous

phase saturated with organic solvents before incubation as shown in Figure 30b. The residual activity of enzyme was observed as 99, 95, 91, 86, 78 and 39% for MTBE, DBE, HEX, DIPE, DEE, and EA, respectively, comparing with the initial activity at the first minute of incubation in each solvent. The stability of *Pe*HNL was observed in the system of solvent log *P* more than 1.5. MTBE, DBE, HEX, and DIPE were suitable solvents for maintaining the enzyme activity (more than 80% of residual activity) during the reaction while the enzyme was unstable during incubation in the system of DEE and EA.

The greatest *e.e.* of 99% was obtained in the biphasic system of EA, DEE, MTBE, DIPE, and DBE (Figure 30a). The system employing HEX as organic phase provided the low *e.e.* of 69% since the undesired racemic products were markedly increased by non-enzymatic reaction. The main reason to describe the different rate of non-enzymatic reaction was partition coefficient of benzaldehyde and HCN releasing from acetone cyanohydrin between organic phase and aqueous phase. Benzaldehyde and HCN are prone to react by non-enzymatic reaction and formed racemic products. To minimize the non-enzymatic reaction, the benzaldehyde should be extracted and separated into the organic phase while the HCN still remains in the aqueous phase and slowly release from acetone cyanohydrin. According to our previous works, the system of DIPE and DEE performed the high values of partition coefficient for partitioning of benzaldehyde to the organic phase. The moderate partition values were found on the systems of DBE, EA, and MTBE, while the lowest partition value was observed on the system of HEX that might causes the acceleration of non-enzymatic reaction significantly.

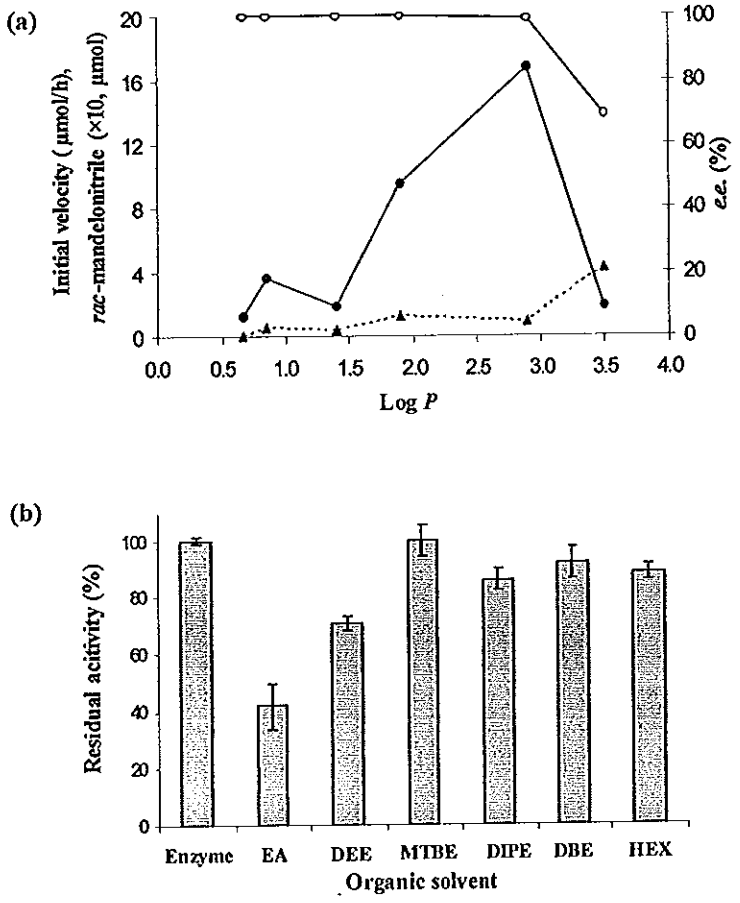


Figure 30. Effect of organic solvent on the transcyanation reaction catalyzed by *Pe*HNL and stability of *Pe*HNL in organic solvents. (a) Effect of *log P* on initial velocity (●), enantiomeric excess (*e.e.*) (○), and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer, pH 4.0, 10 °C, containing 5 U of *Pe*HNL. (b) Effect of organic solvents on stability of *Pe*HNL. (▨) Residual activity of *Pe*HNL after incubated for 12 h or partition coefficient of mandelonitrile.

3.4.4 Aqueous phase content

The content of water is an important parameter in non-aqueous enzymology. In the present study, the reaction catalyzed by *Pe*HNL was conducted in systems of fixed organic phase volume (1 ml) and varied amount of aqueous phase from 5-60% (v/v). The results were shown in Figure 31. The initial velocity of the reaction was influenced by the content of aqueous phase. The initial velocity was increased when increased the aqueous phase from 5-50%, and

fallen down when the aqueous content was more than 50%. The highest initial velocity of the enzyme was observed at 50% aqueous content.

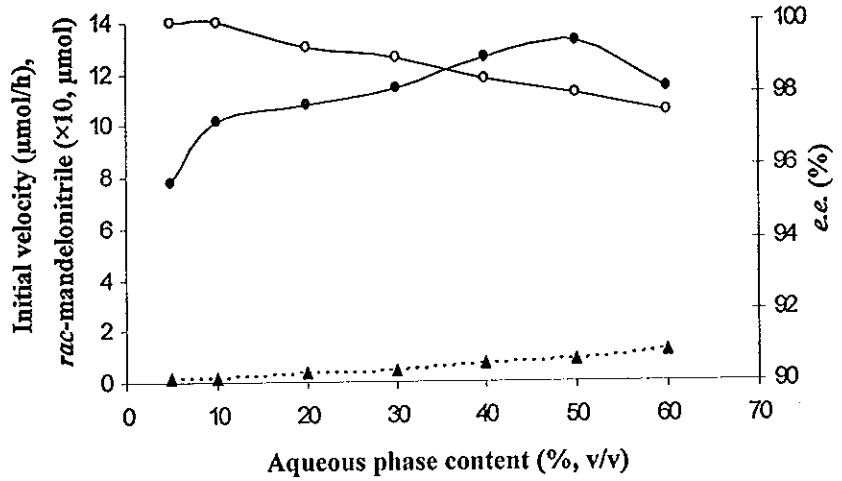


Figure 31. Effect of aqueous phase content on transcyanation reaction catalyzed by *Pe*HNL. Initial velocity (●), enantiomeric excess (*e.e.*) (○), and non-enzymatic reaction (▲) in biphasic system of DBE (1 ml) and buffer (pH 4.0, vary volume of aqueous phase content) at 10 °C containing benzaldehyde (250 mM), acetone cyanohydrin (500 mM), and 5 U of *Pe*HNL.

The increase of initial velocity might be due to the increase of flexibility of enzyme molecules and increase the chance of mass transfer between enzyme and substrate correlated with the increase of aqueous phase content. Moreover, the increase of aqueous phase content might reduce the chance of enzyme inactivation by contacting with the interface of the biphasic system. The aqueous phase content more than the optimum might reduce the reaction of enzyme with the substrate, but less aqueous phase content caused the aggregation of enzyme leading to the lost of initial velocity.

The aqueous phase content slightly influenced the enantiomeric purity of the product. The highest *e.e.* ($\geq$ 99%) was obtained in the system containing 5-30% of buffer, while the *e.e.* was dropped to 97% at the buffer content of 60% caused by the slightly increasing of non-enzymatic reaction when increased the aqueous phase content. The aqueous phase content of 30% was chosen for transcyanation of (*R*)-mandelonitrile to suppress the non-enzymatic background of reaction.

3.4.5 Substrate concentration

The effect of substrate concentration was studied in detail as shown in Figure 32a and 32b. The initial rate of reaction depended on the concentration of substrates. If the amount of acetone cyanohydrin was kept constant (500 mM) and amount of benzaldehyde was gradually increased, the initial velocity increased until it reached an optimum (250 mM of benzaldehyde) (Fig. 32a). After this point, the initial velocity could not be improved as the substrate concentration increased. On the other hand, it was observed that *e.e.* was not affected by the increase of benzaldehyde concentration, and the non-enzymatic reaction was totally suppressed.

As the result in Figure 32b, the initial velocity of *Pe*HNL exhibited a bell shape curve with changing of acetone cyanohydrin concentration and fixing benzaldehyde concentration at 250 mM. The increase of initial velocity was observed from the acetone cyanohydrin concentration of 300-1200 mM. Increasing of the concentration up to 1500 mM caused strong inhibition of the enzyme activity. Adding large amount of acetone cyanohydrin was a point to be concerned. The *e.e.* of 98-99% was obtained when employed acetone cyanohydrin concentration between 250 to 900 mM, but more addition of acetone cyanohydrin caused slightly reduction in *e.e.* (95-97%) due to the incitement of non-enzymatic reaction.

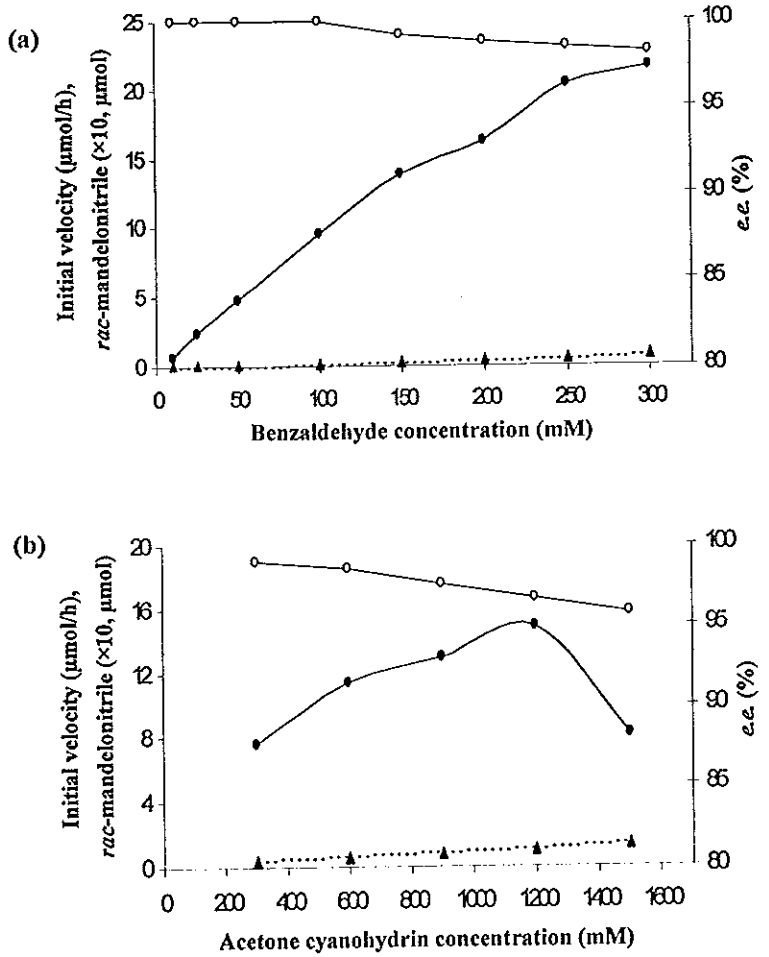


Figure 32. Effect of benzaldehyde (a) and acetone cyanohydrin (b) concentration on transcyanation reaction catalyzed by *Pe*HNL. Initial velocity (●), enantiomeric excess (*e.e.*) (○), and non-enzymatic reaction (▲) in biphasic system of buffer (pH 4.0; 30%, v/v) and DBE at 10 °C. To determine effect of benzaldehyde, concentration of acetone cyanohydrin was kept constantly at 500 mM, while concentration of benzaldehyde was kept at 250 mM when studied on effect of acetone cyanohydrin. The 5 Units of *Pe*HNL was used in all cases.

3.4.6 Enzyme concentration

The effect of enzyme concentrations in the range of 0.67-33.3 units/ml was examined. The initial rate of reaction depended on the concentration of enzyme as illustrated in Figure 33. The constant increase of initial rate was observed when increased the amount of

enzyme up to 26.7 units/ml in the biphasic reaction. Constant initial activity was observed when added enzyme more than 26.7 units/ml.

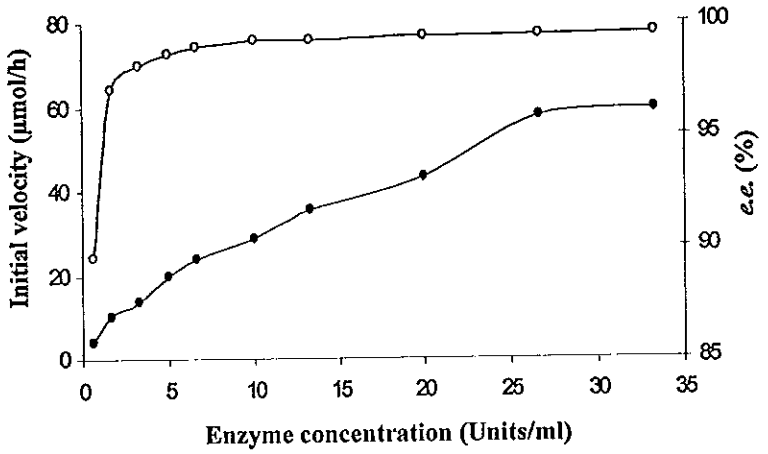


Figure 33. Effect of enzyme concentration on transcyanation reaction catalyzed by *Pe*HNL. Initial velocity (●) and enantiomeric excess (*e.e.*) (○). The reaction was performed in biphasic system of buffer (pH 4.0; 30%, v/v) and DBE, at 10 °C containing benzaldehyde (250 mM), acetone cyanohydrin (900 mM), and various concentration of *Pe*HNL.

The *e.e.* of the product was slightly affected by enzyme concentration. The low concentration of enzyme at 0.67 units/ml caused reduction in the *e.e.* (89.5%). When increase the enzyme concentration, the *e.e.* was increased and reached 99% at the concentration of 6.67 units/ml. Further addition of the enzyme showed no harmful effect on the *e.e.* of product. At the optimum enzyme concentration of 26.7 units/ml reaction, the highest initial velocity of 57.8 µmol/h and *e.e.* of 99.5% were obtained.

3.4.7 Synthesis of (*R*)-mandelonitrile and reusability of *Pe*HNL

The time course of (*R*)-mandelonitrile synthesis by *Pe*HNL was monitored for 24 h and the results are shown in Figure 34a. The linear progress of reaction curve was found in the first hour with 16.1% conversion and *e.e.* of 99.9%. After that the reaction progress curve increased slowly between 1.5-12.0 h with the slightly increase of 26.3% conversion and *e.e.* of 99.5%.

A little increase of the conversion was observed when monitoring the reaction up to 24 h. On the progress curves of (*R*)- and (*S*)-mandelonitrile, (*S*)-mandelonitrile was mostly suppressed in this reaction condition yielding the high *e.e.* along the reaction time.

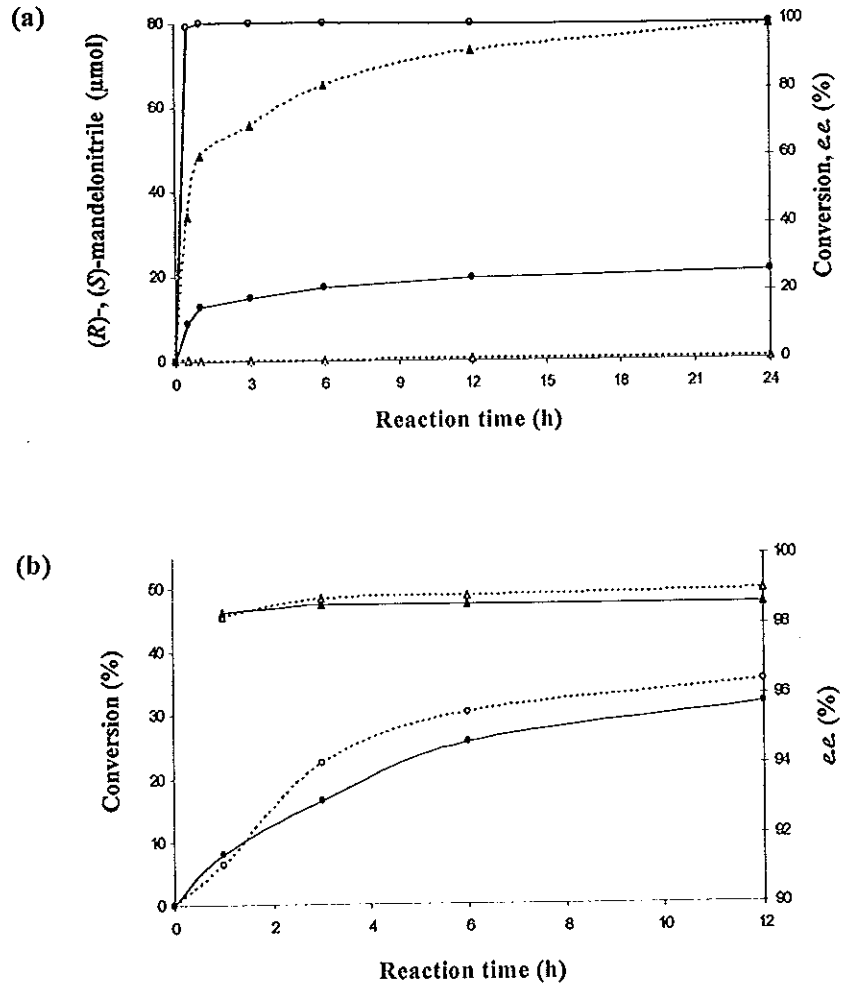


Figure 34. (*R*)-Mandelonitrile synthesis by *Pe*HNL. (a) Time course for transcyanation of (*R*)-mandelonitrile synthesis by *Pe*HNL. The enantiomeric excess (*e.e.*) (—○—), conversion (—●—), (*R*)-mandelonitrile (--▲--) and (*S*)-mandelonitrile (--Δ--) was monitored for 24 h. (b) A comparison of transcyanation of (*R*)-mandelonitrile synthesis by *Pe*HNL and *Pa*HNL. The conversion of *Pe*HNL (—●—) and *Pa*HNL (--○--) and the *e.e.* of *Pe*HNL (—▲—) and *Pa*HNL (--Δ--) was monitored for 12 h. The (*R*)-mandelonitrile synthesis reaction was performed in biphasic system of buffer (pH 4.0; 30%, v/v) and DBE (50%, v/v) at 10 °C containing benzaldehyde (250 mM), acetone cyanohydrin (900 mM), and enzyme (26.7 units/ml).

The comparison of transcyanation of (*R*)-mandelonitrile synthesis by *Pe*HNL and HNL from *Prunus amygdalus* (*Pa*HNL) was performed and demonstrated in Figure 34b. The *e.e.* obtained from *Pe*HNL was 98.6% while that obtained from *Pa*HNL was 98.8%. The conversion of (*R*)-mandelonitrile catalyzed by *Pe*HNL was 31.6%, while the conversion of 35.1% was obtained from the reaction of *Pa*HNL.

The reusability of *Pe*HNL on (*R*)-mandelonitrile synthesis was studied in biphasic system of DBE and buffer. As demonstrated in Table 7, the residual activity of *Pe*HNL in the forth batch was 88.7% of the first batch. The *e.e.* more than 98.5% was obtained in all production batches whereas the conversion slightly decreased.

Table 7. Reusability of *Pe*HNL in repeating batch of (*R*)-mandelonitrile synthesis.

Batch	Initial velocity (μmol/h)	Enantiomeric excess (%)	Conversion (%)
1	59.5	99.5	33.3
2	57.1	99.4	31.7
3	54.3	99.0	28.5
4	52.8	98.7	26.4

3.5 Discussion

A novel (*R*)-hydroxynitrile lyase from *Passiflora edulis* (*Pe*HNL) was discovered by our laboratory. In the previous work, we reported the synthesis of (*R*)-mandelonitrile and (*R*)-methylpropylketone cyanohydrin synthesized by *Pe*HNL with the *e.e.* of 69.4 and 87.0%, respectively (Asano *et al.*, 2005).

In this present study, the successful synthesis of (*R*)-mandelonitrile with high enantiomeric purity employing *Pe*HNL isolated from rind of passion fruit was investigated for the first time. Transcyanation reaction employing acetone cyanohydrin as a cyanide source was used along the experiment because the acetone cyanohydrin has no harmful effect such a free HCN,

water miscible, commercial available and acetone by-product is volatile. Some of the previous works established the analysis methods on HPLC and GC (Klempier and Griengl, 1993; Hanefeld, 2001). These methods contain the derivertization steps of every substance and include several steps which contribute to considerable errors. Moreover, the mandelonitrile concentration was difficult to measure by ^{1}H-NMR method, leading to some problem in the calculation of product conversion (Hickel *et al.,* 1996(b); Kiljunen and Kanerva, 1997). To avoid these problems, the analysis of initial velocity of the reaction, *e.e.* and conversion of the product in our group was carried out by directly analysis of the optically mandelonitrile with chiral HPLC (Asano *et al.,* 2005).

Since the enantiomeric purity or the *e.e.* of 98% of target chiral compound is a minimum acceptable level for commercial process (Pollard and Woodley, 2006) and the initial velocity of the enzyme in the reaction is important, we attempted to achieve the highest *e.e.* of the (*R*)-mandelonitrile and initial velocity of the reaction. The highest *e.e.* of 98.5-99% under the optimized condition was successfully obtained by using biphasic system. Moreover, several parameters influencing on the enantiomeric purity of the products and characteristics of the *Pe*HNL were investigated to achieve the highest enantiomeric purity of product and initial velocity of the reaction.

The pH and temperature affected on several enantioselective enzymes such as nitrile hydratase, amidase, lipase and protease (Yeom *et al.,* 2007; Miyazawa *et al.,* 2006; Lo´pez-Serrano *et al.,* 2001; Sakai, 2004; Wang and Tsai, 2005). The enantiomeric purity of (*R*)-mandelonitrile was mainly affected by the reaction pH and temperature. The optimum pH and temperature giving the highest initial velocity of the reaction was not suitable to obtain the high *e.e.* of the product. To achieve the highest *e.e.*, the low pH and temperature (pH 4 and 10 °C) should be kept along the process to suppress the spontaneous non-enzymatic reaction efficiently. The HNLs from *Prunus amygdalus, Hevea brasiliensis, Manihot exculenta* and *Sorghum bicolor* also peforemed the similar behavior pH and temperature (Costes *et al.,* 1999; Loos *et al.,* 1995; Hickel *et al.,* 2001; Presson *et al.,* 2002).

The reaction of HNLs was occurred at the interface of biphasic system (Cascão-Pereira *et al.,* 2003), therefore the vigorously mix of the reaction mixture to increase the interface area, diffusion and partition of the substrate and product was necessary because mass transfer is

the rate limiting step of reaction (Li *et al.*, 2003, Gerrits *et al.*, 2001). In the previous work on the use of enzyme in organic solvent showed that organic solvent affected on stability and specificity including substrate, stereoselectiviy, regioselectivity, and chemoselectivity of several enzymes (Klibanov, 2001; Broos, 2002). In our study, the initial velocity of reaction was markedly influenced by type of organic solvents, while the *e.e.* of product and stability of the enzyme depend on log *P* of organic solvent. The enantiomeric purity of the product was decreased when performed the reaction in high log *P* organic solvent because of the increase of non-enzymatic reaction. On the contrary, the enzyme had high stability in high log *P* organic solvent, while the lost in residue activity was observed in low log *P* organic solvent. The stability of enzymes in organic solvents depends on the hydrophobicity of the solvent. In general, enzymes only need the thin layer of water on the surface of protein to retain their catalytically active conformation. The most useful non-aqueous media are hydrophobic solvents that do not displace these essential molecules of water from enzymes (Ogino and Ishikawa, 2001; Wong and Whitesides, 1994). The suitable organic solvent should give the highest initial velocity of reaction and enantiomeric purity of the product. Moreover, it should provide the high stability of the enzyme. In our system, DBE was the suitable solvent that reached all criteria. The organic solvents in biphasic system influence the HNLs differently as reported in the previous works, HbHNL was strongly inhibited by EA while PaHNL showed the lowest level of activity in MTBE system comparing with BME (butyl methyl ether), DIPE, DBE, HEP (heptane) (Bauer *et al.*, 1999; Hickel *et al.*, 2001).

Actually, water affects on enzyme stability and catalytic activity. Water is initially interacted with the charged functional group of the protein, subsequently with the uncharged polar and non-polar groups to maintain the active conformation of an enzyme (Wong and Whitesides, 1994; Chen *et al.*, 2001; Han *et al.*, 1998). Besides that the flexibility and activity of enzyme increased with increasing of water content (Broos *et al.*, 1995). Indeed, the optimum of aqueous phase content was influenced by several parameters such as type of enzyme, polarity of the enzyme active site, type of organic solvent, substrate, reaction condition, etc. In the case of *Pe*HNL catalyzed the asymmetric synthesis of (*R*)-mandelonitrile in biphasic system, the greatest *e.e.* of 99% was obtained after optimizing the aqueous phase content, while using the aqueous phase content giving highest initial velocity of reaction caused the reduction of enantiomeric purity. The transcyanation of (*R*)-2-trimethylsilyl-2-hydroxyl-ethylcyanide catalyzed by *Pa*HNL

shown the similar trend of reduction of enantiomeric purity of the product while the initial reaction rate increased when increase the aqueous phase content (Li *et al.*, 2003).

High concentration of substrates and enzyme drives the reaction thermodynamically. At the saturation of substrates around the active site of the enzyme, the increase of initial velocity of the enzyme was slow or the enzyme might be inhibited by the substrates leading to the decrease of initial velocity of reaction. Furthermore, increase of substrate concentration caused slightly reduction of enantiomeric purity.

In this study, the enzyme concentration was optimized to obtain the highest enantiomeric purity of product and initial velocity of the reaction whereas the previous experiment reported elsewhere employed the excess amount of enzyme in the synthesis to achieved high enantiomeric purity but neglected to measure the initial activity and optimize the amount of enzyme (Lin *et al.*, 1999; Kiljunen and Kanerva, 1996; Hanefeld *et al.*, 2001). The optimum enzyme concentration should be used to obtain the great initial velocity and enantiomeric purity, besides that the lowest cost of reaction might be achieved.

3.6 Conclusion

The transcyanation of (*R*)-mandelonitrile synthesis by a novel *Pe*HNL was successfully demonstrated in this present study for the first time. It is necessary to study and optimize all parameters to obtain the high initial velocity of reaction and enantiomeric purity of the chiral product, due to several parameters influenced on the reaction complicatedly. Moreover, the appropriate biphasic system for synthesis the (*R*)-mandelonitrile with high enantiomeric purity in our study showed the reusability of enzyme and easily to recover the product in downstream process.

CHAPTER 4

Purification and characterization of a novel (*R*)-hydroxynitrile lyase from *Eriobotrya japonica* (loquat)

4.1 Abstract

A hydroxynitrile lyase has been isolated and purified to homogeneity from seeds of *Eriobotrya japonica* (loquat). The final yield of 36% with 50-fold purification was obtained by 30-80% $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-Toyopearl and Concanavalin A Sepharose 4B, which suggested the presence of a carbohydrate side chain. The purified enzyme is a monomer with a molecular mass of 72 kDa as determined by gel filtration and 62.3 kDa as determined by SDS-gel electrophoresis. The enzyme is a flavoprotein containing FAD as a prosthetic group and exhibits a K_m of 161 μM and k_{cat}/K_m of 348 $sec^{-1}mM^{-1}$ for mandelonitrile. The optimum pH and temperature were pH 5.5 and 40 °C, respectively. The enzyme showed excellent stability with regard to pH (pH 3-9) and temperature (0-60 °C). Metal ions were not required for its activity, while the activity was significantly inhibited by $CuSO_4$, $HgCl_2$, $AgNO_3$, $FeCl_3$, β-mercaptoethanol, iodoacetic acid, phenylmethylsulfonylfluoride, and diethylpyrocarbonate. The specificity constant (k_{cat}/K_m) of the enzyme was investigated for the first time using various aldehydes as substrates. The enzyme was active toward aromatic and aliphatic aldehydes and showed a preference for smaller substrates over bulky substrates.

4.2 Introduction

Hydroxynitrile lyase (HNL) is one of the enzymes involved in the catabolism of cyanogenic glycosides in higher plants. This enzyme catalyses the decomposition of cyanohydrins into the corresponding aldehydes or ketones and HCN for plant defense against predators and microorganisms (Vetter, 2000). The reverse reaction of HNL, which is synthesis of chiral cyanohydrins, has attracted the attention of scientists and industries. Chiral cyanohydrins, alcohols containing a cyano group attached to the same carbon atom, are versatile building blocks in the synthesis of large numbers of biologically active compounds in fine chemicals, pharmaceuticals, veterinary products, crop-protecting agents, vitamins, and food additives (Kruse, 1992). HNL activity was detected for the first time in kernels of *Prunus dulcis (amygdalus)* or almond by Friedrich Wöhler in 1837, cleaving the cyanohydrins to aldehyde and HCN (Wöhler and Liebig, 1837). The synthesis of chiral cyanohydrins by HNL from almond was first reported by Rosenthaler (Rosenthaler, 1980).

Recently, we developed a new screening method using chiral HPLC to determine the activity and stereoselectivity of HNL (Asano *et al.*, 2005). We discovered several novel sources of HNL among 163 plant species in 74 families examined. (*S*)-HNL activity was found in a homogenate of the leaves of *Baliospermum montanum*, while (*R*)-HNLs were detected in homogenates of the leaves and seeds of *Passiflora edulis*, and seeds of *Eriobotrya japonica*, *Chaenomeles sinensis*, *Sorbus aucuparia*, *Prunus mume*, and *Prunus persica*.

Eriobotrya japonica (Thunb.) Lidnley, also known as Japanese medlar, is an evergreen tree of the Maloideae subfamily of the Rosaceae family that is native to southeastern China, and has long been introduced to Japan, hence its name. It is cultivated as well as in various tropical and subtropical countries (Femenia *et al.*, 1998). Its golden fruits, loquat, is round or oval in shape and has a sweet taste. The fruit is eaten fresh or made into preserves, jam, jelly, juice, and nectar after removal of the seeds as waste (Ibarz *et al.*, 1995). Seeds of *Eriobotrya* L. were reported to be a source of (*R*)-hydroxynitrile lyase for the synthesis of cyanohydrins (Lin *et al.*, 1999) However, the researches on the enzymatic synthesis of chiral cyanohydrins synthesis have mostly been carried out using an excess amount of crude enzyme and a long reaction time to

obtain high enantiomeric excess (*e.e.*) and conversion. Crude enzyme or seed powder was directly used for the reaction without purification and characterization of the enzyme (Lin *et al.*, 1999; Kiljunen and Kanerva, 1996; Kiljunen and Kanerva, 1997). In the case of HNL from *E. japonica* (*Ej*HNL), lack of information and the understanding of the characteristics of purified enzyme lead to limit the enzyme applications.

In order to understand the characteristics of *Ej*HNL, the isolation, purification, and characterization of HNL from seeds of *E. japonica* as well as its application in the synthesis of cyanohydrins were studied.

4.3 Materials and Methods

4.3.1 Materials

Seeds of *E. japonica* were purchased from the National Federation of Agricultural Co-operative Associations (Nagasaki, Japan) and stored at 4 °C. All chemicals used in the experiments were purchased from commercial sources and used without further purification. Silica gel TLC plates (Merck & Co., Inc., New Jersey, USA) were used to follow the chemical reaction. Silica gel 100-200 mesh (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for column chromatography. NMR spectra were recorded with a JEOL LA-400 spectrometer (Tokyo, Japan) at 25 °C in $CDCl_3$ using TMS as the internal standard. Chemical shifts are shown as δ_H and δ_C for ^1H and ^{13}C NMR spectra, respectively. Chiral HPLC was performed using a chiralcel OJ-H column (Diacel Chemical Industries Ltd., Osaka, Japan) with a SPD-10A VP UV-vis detector (Shimadzu, Kyoto, Japan) at 254 nm. The eluting solvent was 90% hexane and 10% isopropanol by volume. A chiral GC analysis was performed using a GAS CHROMATOGRAPH GC-14B (Shimadzu, Kyoto, Japan) with a fused silica capillary β-Dex-325 column (SUPELCO, Pennsylvania, USA) and He as a carrier gas (Detector temperature: 230 °C, injection temperature 220 °C).

4.3.2 Crude enzyme extraction

Seeds of *E. japonica* were sterilized by soaking in 0.1% (v/v) sodium hypochlorite and rinsed with de-ionized water. All purification steps were carried out at 4 °C. Sterilized seeds were homogenized with 3% polyvinylpyrolidone in 10 mM potassium phosphate buffer, pH 6.0 (100 ml/ 100 g fresh seeds) in a SMT Process Homogenizer, PH91 (SMT Company, Tokyo, Japan). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 28,000 × *g* and 4 °C for 30 min. The supernatant was used as the crude enzyme extract.

4.3.3 Ammonium sulfate precipitation

The crude extract was fractionated by 0-30, 30-80% saturation of ammonium sulfate. The precipitate fractionated at 30-80% was collected by centrifugation at 28,000 × *g* and 4 °C for 30 min. The precipitate was dissolved and dialyzed overnight against an excess volume of the same buffer. Any precipitate formed during dialysis was removed by centrifugation and the supernatant was collected.

4.3.4 DEAE-Toyopearl 650M column chromatography

The enzyme solution was loaded onto a DEAE-Toyopearl 650M column (26 mm×10.5 cm, 50 ml) equilibrated with 10 mM potassium phosphate buffer, pH 6.0 and eluted with a linear gradient of NaCl (0-0.5 M). Fractions of 2 ml were collected. The protein profile was monitored by measuring absorbance at 280 nm, the protein fractions were assayed for HNL activity, and the active fractions were pooled for further analysis.

4.3.5 Concanavalin A Sepharose 4B column chromatography

The enzyme solution was purified on a Concanavalin A Sepharose 4B column (5 mm×5 cm, 1 ml) equilibrated with 0.5 M NaCl in 10 mM potassium phosphate buffer, pH 6.0 and

eluted with linear gradient of 0-1 M of α-D-methylglucoside. Fractions of 1 ml were collected. Protein profile was monitored by measuring the absorbance at 280 nm and the protein fractions were assayed for HNL activity.

4.3.6 HNL activity assay

HNL activity was measured by monitoring the decomposition of (*R,S*)-mandelonitrile to benzaldehyde according to the method described by Willeman (Willeman *et al.*, 2000) with a slight modification. The conversion of (*R,S*)-mandelonitrile to benzaldehyde was followed by continuously measuring the increase in absorbance at 280 nm. The reaction was performed in a quartz cell. The enzyme solution was added to a 50 mM sodium citrate phosphate buffer, pH 5.5, containing 2 mM mandelonitrile in a total volume of 1 ml. Then, the reaction mixture was mixed gently and the reaction was followed for 5 min by spectrophotometer at 280 nm (U-3210 spectrophotometer, Hitachi, Japan). The linear change in absorbance for the initial 1 min was used for the calculation. The slope of absorbance was determined and subtracted with a blank. By using ε_{280}=1.4 $mM^{-1}cm^{-1}$, the enzyme activity was calculated.

One unit of HNL activity (decomposition unit) is defined as the amount of enzyme that converted 1 μmol of mandelonitrile to benzaldehyde in 1 min under standard assay conditions. According to a previous study on *Ej*HNL by our group, one unit of HNL activity (synthetic unit) was defined as the amount of enzyme that produced 1 μmol of optically active mandelonitrile from benzaldehyde per min under the described assay condition (Asano *et al.*, 2005). The decomposition activity of *Ej*HNL is about 1.3 times more than the synthetic activity.

4.3.7 Protein assay

Protein concentrations were measured using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard (Bradford, 1976).

4.3.8 Gel electrophoresis

SDS-PAGE and Native PAGE were used to analyze the molecular mass and homogeneity of the enzyme, respectively. The gel electrophoresis was performed with a 1-mm-thick polyacrylamide gel (Laemmli, 1970). Standard protein markers for SDS-PAGE consist of phosphorylase *b* (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da) and lysozyme (14,400 Da).

4.3.9 Estimation of apparent molecular mass

The molecular mass of *Ej*HNL was estimated by means of gel-filtration chromatography on a Superdex-200 column HR 10/30 (10 mm×30 cm, 24 ml). The purified enzyme was loaded onto the column and eluted at a flow rate of 0.2 ml/min with 10 mM potassium phosphate buffer, pH 6.0 containing 0.15 M NaCl. The standard protein markers used for calibration were as follows: thyroglobulin (670,000 Da), γ-globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da), and vitamin B2 (13,500 Da).

4.3.10 N-terminal sequence

The N-terminal sequence of the purified enzyme (40 μl) was sequenced (30 cycles) with a HP G1005A Protein Sequencing system by APRO Science (Tokushima, Japan).

4.3.11 Prosthetic group analysis

Absorption spectra were measured by a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan), using a 1-cm cell pathlength. The enzyme was analyzed in 10 mM potassium phosphate buffer, pH 6.0.

4.3.12 Kinetic parameters

The initial velocity of the enzymatic decomposition of racemic mandelonitrile was determined in 50 mM sodium citrate buffer, pH 5.5 according to the standard reaction assay with various substrate concentrations.

4.3.13 Effects of pH and temperature

The optimum pH and temperature for activity were assayed according to standard method at pH 3.5-6.5 and temperature 10-80 °C. Stability was monitored after 60 min incubation at various pH (3-9, 30 °C) and temperatures (0-80 °C, pH 6.0).

4.3.14 Effect of additives

The effect of various additives on the purified enzyme was examined. The enzyme solution was incubated with various additives in 10 mM potassium phosphate buffer, pH 6.0 at 30 °C for 60 min. The remaining activity of the enzyme was assayed according to the standard procedure.

4.3.15 Synthesis of cyanohydrins

The reaction mixture was prepared in a micro-tube: 1.0 M of carbonyl compound (in DMSO, 40 µl) was added to sodium citrate buffer (400 mM, pH 4.0, 760 µl), followed by the addition of enzyme solution (25 units, decomposition units) and KCN solution (1.0 M, 100 µl). The reaction was monitored by taking a small aliquot of the reaction mixture (100 µl) and extracting with organic solvent (90% n-hexane and 10% isopropanol by volume for HPLC, ethyl acetate for GC). The organic layer was analyzed by chiral HPLC (for aromatic compounds) and chiral GC (for aliphatic compounds).

To calculate the kinetic parameters of the enzymatic synthesis of cyanohydrins, the initial velocity of the synthesis of chiral cyanohydrins was determined under the conditions described above with various aldehyde substrate concentrations.

4.3.16 Preparation of cyanohydrins for HPLC and GC standard

The carbonyl compound (1 eq.) was dissolved and stirred vigorously in acetic acid. This was followed by the addition of an aqueous solution of KCN (3 eq.) into the mixture. The reaction was monitored by TLC with UV-light or iodine vapor as a developing agent. After the reaction was completed, the reaction mixture was neutralized with $NaHCO_3$, extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in a vacuum. The cyanohydrins after purification by silica gel column chromatography were characterized by ^1H and ^{13}C-NMR spectroscopy and used as standards for HPLC and GC. All cyanohydrins isolated were colorless to slightly yellow oils.

4.3.17 NMR data of standard cyanohydrins

***2b*: 2-hydroxy-2-(4-methoxyphenyl)acetonitrile (*p*-anisaldehyde cyanohydrin)**

$C_9H_9O_2N$; ^1H NMR δ_H ($CDCl_3$): 7.4 (d, 2H, Ar-*H*), 6.9 (d, 2H, Ar-*H*), 5.4 (d, 1H, C*H*CNOH), 3.8 (s, 3H, CH_3O-Ar), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 160.8, 128.3, 127.5, 118.8, 114.5, 63.4, 55.4

***2c*: 2-hydroxy-2-(thiophen-2-yl)acetonitrile (2-thiophene carboxaldehyde cyanohydrin)**

C_6H_5ONS; ^1H NMR δ_H ($CDCl_3$): 7.0-7.5 (m, 3H, Ar-*H*), 5.7 (s, 1H, C*H*CNOH), 2.7 (s, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 148.0, 128.1, 127.4, 127.2, 118.2, 59.36

***2d*: 2-hydroxy-2-(naphthalen-1-yl)acetonitrile (1-naphthaldehyde cyanohydrin)**

$C_{12}H_9ON$; ^1H NMR δ_H ($CDCl_3$): 7.5-8.1 (m, 7H, Ar-*H*), 6.1 (d, 1H, C*H*CNOH), 2.7 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 134.1, 131.1, 130.4, 130.0, 129.1, 127.4, 126.6, 125.7, 125.1, 122.9, 118.0

***2e*: 2-hydroxy-2-(naphthalen-2-yl)acetonitrile (2-naphthaldehyde cyanohydrin)**

$C_{12}H_9ON$; ^1H NMR δ_H ($CDCl_3$): 7.4-8.0 (m, 7H, Ar-*H*), 6.0 (d, 1H, C*H*CNOH), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 135.2, 133.7, 131.8, 128.0, 127.6, 127.5, 127.3, 127.0, 126.0, 125.1, 118.2, 63.3

***2f*: 2-(benzo[*d*][1,3]dioxol-6-yl)-2-hydroxyacetonitrile (piperonaldehyde cyanohydrin)**

$C_9H_7O_3N$; ^1H NMR δ_H ($CDCl_3$): 7.0 (dd, 2H, Ar-*H*), 6.8 (dd, 1H, Ar-*H*), 6.0 (s, 2H, -OCH_2O-), 5.4 (d, 1H, C*H*CNOH), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 148.9, 148.4, 129.1, 120.7, 118.6, 108.6, 107.2, 101.6, 63.5

***2g*: 2-hydroxybutanenitrile (propionaldehyde cyanohydrin)**

C_4H_7ON; ^1H NMR δ_H ($CDCl_3$): 4.4 (t, 1H, C*H*CNOH), 2.9 (s, 1H, O*H*), 1.8 (qd, 2H, CH_2), 1.1 (t, 3H, CH_3). ^{13}C NMR δ_C ($CDCl_3$): 119.8, 62.5, 28.6, 8.9

***2h*: 2-hydroxy-3-methylbutanenitrile (isobutyraldehyde cyanohydrin)**

C_5H_9ON; ^1H NMR δ_H ($CDCl_3$): 4.3 (m, 1H, C*H*CNOH), 2.8 (s, 1H, O*H*), 2.1 (m, 1H, C*H*), 0.9 (m, 6H, CH_3). ^{13}C NMR δ_C ($CDCl_3$): 118.4, 67.0, 33.0, 17.6, 17.1

***2i*: 2-hydroxy-3,3-dimethylbutanenitrile (pivalaldehyde cyanohydrin)**

$C_6H_{11}ON$; ^1H NMR δ_H ($CDCl_3$): 4.1 (d, 1H, C*H*CNOH), 2.4 (d, 1H, O*H*), 1.0 (s, 9H, CH_3). ^{13}C NMR δ_C ($CDCl_3$): 118.4, 70.7, 35.4, 24.9

***2j*:2-cyclohexyl-2-hydroxyacetonitrile (cyclohexanecarboxaldehyde cyanohydrin)**

$C_8H_{13}ON$; ^1H NMR δ_H ($CDCl_3$): 4.2 (t, 1H, C*H*CNOH), 2.5 (d, 1H, O*H*), 1.8 (m, 10H, CH_2), 1.2 (m, 1H, C*H*). ^{13}C NMR δ_H ($CDCl_3$): 119.2, 66.4, 42.2, 30.9, 28.1, 27.7, 25.9, 25.4

4.4 Results

The hydroxynitrile lyase from seeds of *Eriobotrya japonica* (*Ej*HNL) was purified to homogeneity. The results for a typical preparation are summarized in Table 8.

Table 8. Purification of hydroxynitrile lyase from *Eriobotrya japonica*.

Purification step	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)	Purification (fold)
Crude extract	840	1000	0.8	100	1
30-80% $(NH_4)_2SO_4$	522	140	3.7	62	4
DEAE-Toyopearl 650M	369	38.3	9.7	44	12
Concanavalin A Sepharose 4B	303	7.5	40.9	36	50

Crude enzyme with 840 units of activity was extracted from 2 kg of seeds. Ammonium sulfate precipitation and salting out were used to concentrate the crude enzyme solution before the column chromatography. The *Ej*HNL was precipitated between 30-80% saturated salt solution, approximately 62% of the activity remained and 4-fold purification was obtained by the salt precipitation step.

The re-dissolved enzyme solution was further purified by anion-exchange chromatography on a column of DEAE-Toyopearl 650 M. Proteins absorbed on the column were eluted by increasing the linear gradient from 0-0.5 M NaCl. Fractions having HNL activity were combined for further purification. Specific activity increased approximately three fold.

An active pool of DEAE-Toyopearl fractions was dialyzed against 10 mM potassium phosphate buffer, pH 6.0. Prior to being loaded onto the affinity column, Concanavalin A Sepharose 4B, the enzyme solution was mixed with NaCl to obtain a concentration of 0.5 M. After loading and washing, the column was eluted with 0-1 M of α-D-methylglucoside as a linear gradient. One major symmetrical peak of protein was eluted approximately at 0.2 M of α-D-methylglucoside. Large amounts of contaminated proteins were removed. With this step, the

purification of 50 folds with a yield of 36 % was obtained. The enzyme was interacted with concanavalin A column indicating the enzyme is a glycoprotein.

The native molecular mass of purified *Ej*HNL was estimated by HPLC on a Superdex 200 filtration column. From logarithmic plots of the molecular mass *vs.* retention time of standard proteins, the molecular mass of the native enzyme was estimated to be 72 kDa. The purified enzyme was appeared as one band on the native-PAGE gel (Figure 35a) with a molecular mass of 62.3 kDa from the SDS-PAGE gel (Figure 35b). Consequently, it was concluded that the purified enzyme consisted of a monomer of a single subunit. A thick purified band in SDS-PAGE might be caused by the glycosidic properties of the enzyme.

The yellow color of the *Ej*HNL was also observed during the purification and the enzyme exhibited maximum absorption spectra at 278, 389, and 454 nm which are seen in the absorption spectra of a typical FAD containing protein. The N-terminal sequence of the purified *Ej*HNL was L-A-T-P-S-E-H-D-F-S-Y-S-K-S-V-V-X-A-T-D-L-P-Q-E-E-V-Y-D. The symbol X represents an unidentified amino acid. The 57 % identity in 28 amino acids was found to overlap with the sequence of the FAD-containing (*R*)-hydroxynitrile lyase from *Prunus serotina* (Cheng and Poulton, 1993) and *Prunus dulcis* (Dreveny *et al.,* 2001) but different from non-FAD hydroxynitrile lyases. Therefore, the *Ej*HNL was indicated to be the FAD containing HNL.

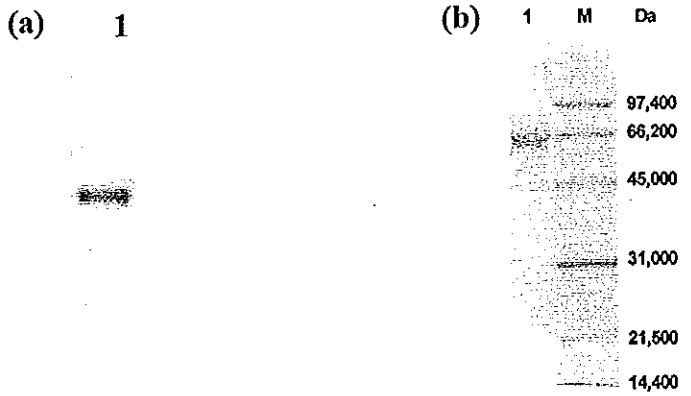


Figure 35. Native-PAGE (a) and SDS-PAGE (b) of purified *Ej*HNL. M: Low molecular weight standard marker; lane 1: purified *Ej*HNL.

A substrate saturation curve for the purified *Ej*HNL was determined using mandelonitrile. As shown in Figure 36, the typical Michaelis-Menten kinetics exhibited a K_m of 161 μM, k_{cat} of 56 sec^{-1}, and k_{cat}/K_m of 348 $sec^{-1}mM^{-1}$.

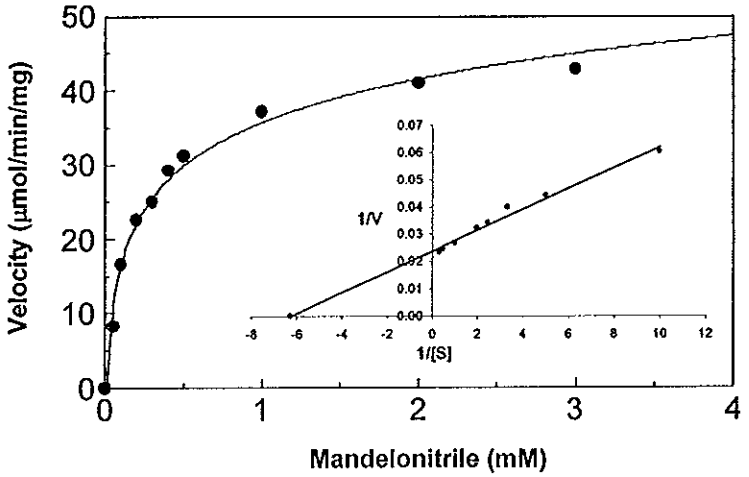


Figure 36. Substrate saturation curve for mandelonitrile with *Ej*HNL. The assay condition were described in Materials and methods, the concentration of mandelonitrile was varied as indicated. The inset is Lineweaver-Burk plot, V_{max} of 46.5 μmol/min/mg, K_m value of 161 μM, k_{cat} 56 sec^{-1} and k_{cat}/K_m 348 $sec^{-1}mM^{-1}$ were obtained.

The effect of pH on the enzyme activity is shown in Figure 37. The optimum pH of *Ej*HNL using the natural substrate mandelonitrile was found to be approximately 5.5. At pH 3.5, the activity was totally inhibited but a rapid increase activity was found on increasing the pH up to the optimum. At a higher pH than the optimum, an increase in the spontaneous decomposition of the substrate was observed, therefore the actual optimum pH was difficult to determine (Asano *et al.*, 2005). After incubation at various pH for 60 min, excellent stability was found at pH 3-9 (data not shown). At pH 5.5, the optimum temperature was 40 °C with mandelonitrile as a substrate (Figure 38). Temperature lower and higher than the optimum caused a decrease in activity. Besides the heat-inactivation effect, high temperature was found to be a cause of the spontaneous decomposition of the substrate which affected the enzyme activity. The temperature stability was studied, *Ej*HNL was found to be active over a broad range of temperatures (0-60 °C) after incubation for 60 min. At 70 and 80 °C, the residual activities of the enzyme after incubation for 60 min were 68 and 28 %, respectively (data not shown). At 4 °C in

10 mM potassium phosphate buffer (pH 6.0), *Ej*HNL was stable for at least 30 days without any significant loss of activity.

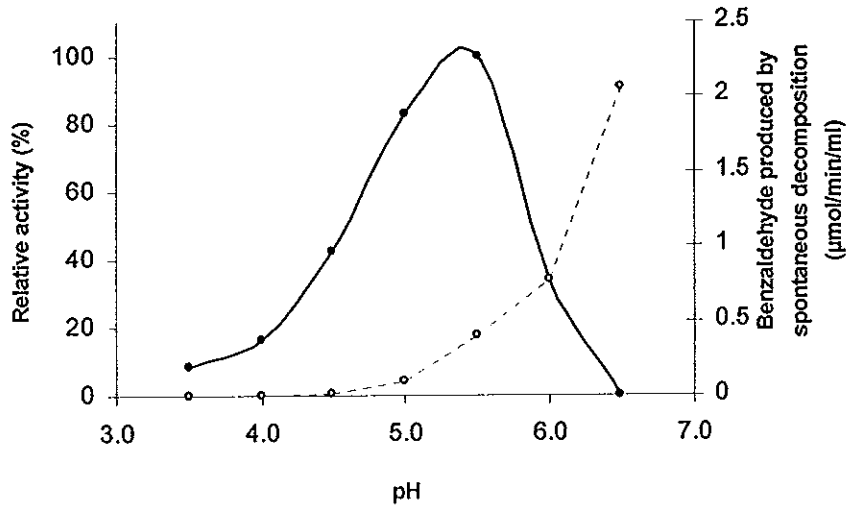


Figure 37. pH profiles of purified *Ej*HNL and non-enzymatic decomposition of mandelonitrile. (●) Enzyme activity, (○) Spontaneous decomposition of mandelonitrile.

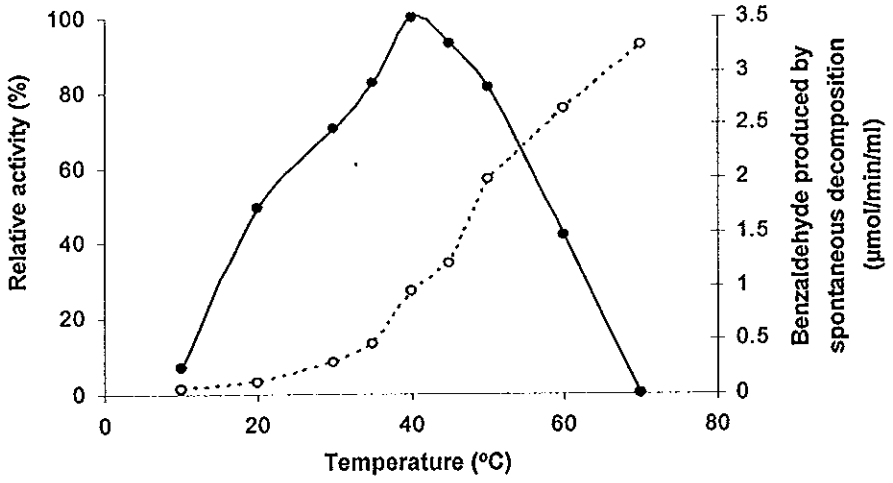


Figure 38. Temperature profiles of purified *Ej*HNL and non-enzymatic decomposition of mandelonitrile. (●) Enzyme activity, (○) Spontaneous decomposition of mandelonitrile.

Next the influences of various additives on the activity of *Ej*HNL were determined (Table 9). The rate of degradation of mandelonitrile by *Ej*HNL was unaffected by 1 mM of $ZnCl_2$, $MnCl_2$, $MgCl_2$, or $PbCl_2$, and by 10 mM of Na_2EDTA, while 1 mM of $FeCl_3$ caused 50% inhibition. These results suggested that metal ions were not required for the activity. The enzyme was significantly inhibited by a reducing agent and cysteine modifying agent. β-Mercaptoethanol caused 19% inhibition at 10 mM, iodoacetic acid caused 53% inhibition at 1 mM, while $CuSO_4$, $HgCl_2$, and $AgNO_3$ caused complete inhibition. The serine modifying agent phenylmethylsulfonylfluoride (PMSF) inhibited the enzymatic activity by 59% at 1 mM, while the histidine inhibitor diethylpyrocarbonate (DEP) caused 36% inhibition at 1 mM. These results indicated that cysteine, serine, and histidine residues are located near the active site of the enzyme and participate in the catalysis (Dreveny *et al.*, 2002).

Table 9. Effect of various additives on the activity of purified *Ej*HNL.

Additive	Concentration (mM)	Inhibition (%)
β-Mercaptoethanol	10	19
Iodoacetic acid	1	53
PMSF	1	59
DEP	1	36
Na_2EDTA	10	0
$MnCl_2$	1	0
$ZnCl_2$	1	0
$MgCl_2$	1	0
$CuSO_4$	1	100
$HgCl_2$	1	100
$PbCl_2$	1	0
$AgNO_3$	1	100
$FeCl_3$	1	50

The synthesis of chiral cyanohydrins from selected aromatic and aliphatic aldehydes (Figure 39) by *Ej*HNL was investigated as shown in Table 10.

A specificity constant (k_{cat}/K_m) was used to compare the relative rate of the enzyme acting on substrates. For aromatic aldehydes, a high specificity constant was obtained for 2-thiophenaldehyde, benzaldehyde and 2-naphthaldehyde at 160.5, 148.4, and 101.3 $sec^{-1}mM^{-1}$, respectively. The specificity constant for bulky aromatic substrates, *p*-anisaldehyde and piperonal, was 50.8 and 29.2 $sec^{-1}mM^{-1}$, respectively, while 1-naphthaldehyde could not be catalyzed by the enzyme. In contrast to the report on the substrate specificity of HNL from *Eriobotrya* L. by Lin *et al.* (1999) that aliphatic aldehyde (trimethylacetaldehyde) was an unacceptable substrate for the enzyme, we found that *Ej*HNL acted on propionaldehyde, pivaldehyde and isobutyraldehyde with a specificity constant of 19.4, 14.3, and 9.6 $sec^{-1}mM^{-1}$, respectively. Cyclohexanecarboxaldehyde was a poor substrate for *Ej*HNL.

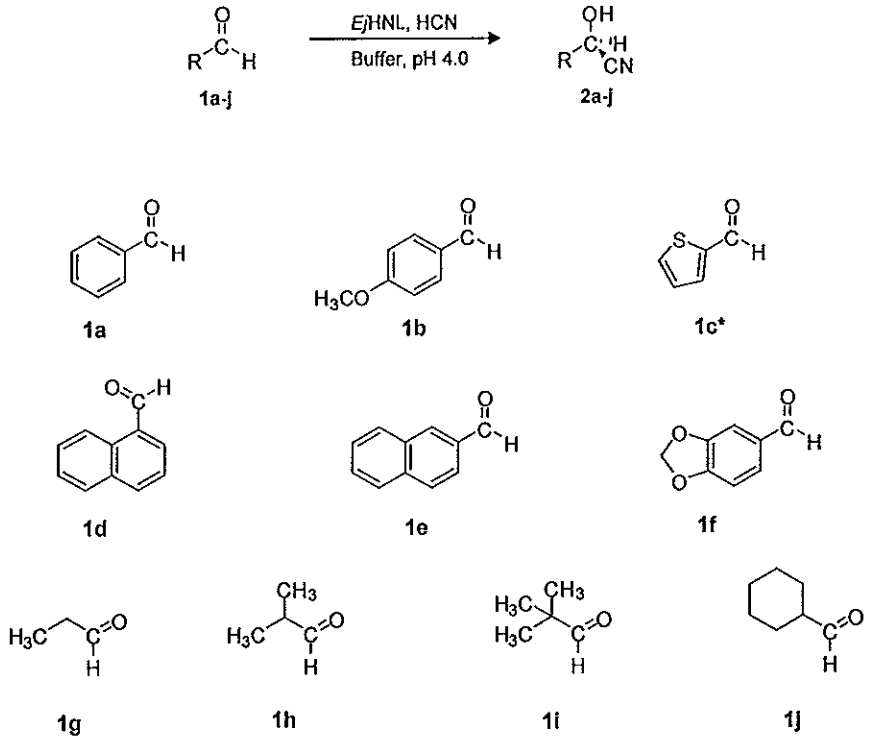


Figure 39. Synthesis reaction of asymmetric (*R*)-cyanohydrins catalyzed by (*R*)-*Ej*HNL and aldehyde substrates. *(*S*)-configuration was assigned according to Cahn-Ingold-Prelog (Eliel *et al.*, 1994).

Table 10. Cyanohydrins synthesis and substrate specificity of *Ej*HNL on aromatic and aliphatic aldehydes

Entry	Substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1}sec^{-1}$)	*e.e.* (%)	Configuration
1a	Benzaldehyde	11.5	1700	148	65	*R*
1b	*p*-Anisaldehyde	3.5	582	50.8	81	*R*
1c	2-Thiophenaldehyde	35.7	1840	161	90	*S*[a]
1d	1-Naphthaldehyde	-	-	-	24	*R*
1e	2-Naphthaldehyde	1.1	1160	101	88	*R*
1f	Piperonal	2.6	334	29.2	89	*R*
1g	Propionaldehyde	99.9	223	19.4	36	*R*
1h	Isobutyraldehyde	41.8	110	9.6	38	*R*
1i	Pivaldehyde	39.3	164	14.3	14	*R*
1j	Cyclohexane carboxaldehyde	-	-	-	4	*R*

[a] (*S*)-configuration was assigned according to Cahn-Ingold-Prelog (Eliel *et al.*, 1994).

The enantiomeric excess (*e.e.*) of chiral cyanohydrins on different substrates was studied (Table 10). The *e.e.* was in the range of 3.9-89.5%. Two factors might have affected on the *e.e.* value; the specificity of the enzyme for the substrates used and the rate of spontaneous chemical reaction. The high *e.e.* of chiral cyanohydrins could be achieved with biphasic systems of buffers and water-immiscible organic solvents, and the spontaneous chemical reaction could be suppressed under these systems (Asano *et al.*, 2005). These results will be described elsewhere.

4.5 Discussion

HNL was originally classified based on enantioselectivity into two groups. (*R*)-selective HNL catalyzes the formation of (*R*)-cyanohydrins and almost derived from oxidoreductase ancestors such as HNLs from *Rosaceae* and *Linum usitatissimum*, except for an (*R*)-selective HNL from *Arabidopsis thaliana* is derived from an α/β-hydrolase fold (Wajant and Effenberger, 1996; Andexer *et al.*, 2007). (*S*)-selective HNL, derived from hydrolases with an α/β-hydrolase fold, catalyzes the formation of (*S*)-cyanohydrins such as HNLs from *Hevea brasiliensis, Manihot esculenta* (Wajant and Effenberger, 1996).

However, according to the EC number based on the chemical reaction of the enzyme, HNL can be classified into 4 groups. Firstly, mandelonitrile lyase (EC 4.1.2.10) is a (*R*)-HNL that converts the natural substrate (*R*)-mandelonitrile to benzaldehyde and prussic acid. Mandelonitrile lyases from the Rosaceae family (Prunoideae and Maloideae subfamilies) are glycoproteins with posttranslational modifications and contain FAD as a prosthetic group. Several plants containing this type of HNL were reported including *Prunus amygdalus* (Worker *et al.*, 1994), *P. serotina* (Hu and Poulton, 1999), *P. mume* (Asano *et al.*, 2005), *P. persica* (Asano et al., 2005; Hernández *et al.*, 2004), *P. avium* (Hernández *et al.*, 2004), *P. domestica* (Hernández *et al.*, 2004), *P. laurocerasus* (Gerstner and Kiel, 1975), *P. lyonii* (Xu *et al.*, 1986), *Passiflora edulis* (Asano *et al.*, 2005), *Chaenomles sinensis* (Asano *et al.*, 2005), *Pouteria sapota* (Hernández *et al.*, 2004), *Cucumis melo* (Hernández *et al.*, 2004), *Cydonia oblonga* (Hernández *et al.*, 2004), and *Phlebodium aureum* (Wajant *et al.*, 1995). Secondly, *p*-hydroxymandelonitrile lyase (EC 4.1.2.11) is a (*S*)-HNL that catalyzes the decomposition of (*S*)-mandelonitrile and (*S*)-4-hydroxymandelonitrile as natural substrates. *p*-Hydroxymandelonitrile lyases have been investigated in plant extracts of *Baliospermum montanum* (Asano *et al.*, 2005), *Sorghum bicolor* (Wajant *et al.*, 1993), *Annona cherimola* (Hernández *et al.*, 2004), *Annona squamosa* (Hernández *et al.*, 2004), and *Ximenia americana* (Kuroki and Conn, 1989). Thirdly, acetone cyanohydrin lyase (EC 4.1.2.37) is a (*R*)-HNL that catalyzes the degradation of acetone cyanohydrin in *Linum usitatissimum* (Albrecht *et al.*, 1993). Lastly, hydroxynitrilase (EC 4.1.2.39), a (*S*)-HNL

catalyzing the decomposition of acetone cyanohydrin and 2-butanone cyanohydrin was found in *Hevea brasiliensis* (Wajant and Förster, 1996) and *Manihot exculenta* (Hughes *et al.*, 1994).

Previous studies on the purification and characterization of hydroxynitrile lyases investigated only some characteristics such as molecular mass, optimum pH and temperature, effects of additives and inhibitors, storage effects, etc. A description of all characteristics including substrate specificity based on activity was unclearly described. Seeds of *Eriobotrya japonica* were found to be an interesting source of HNL (Asano *et al.*, 2005). Ground seeds of *Eriobotrya L.* were used directly to synthesize chiral cyanohydrins without further purification and study the characteristics of the enzyme (Lin *et al.*, 1999). Lack of information or understanding of the real characteristics of purified enzymes limits their application. Therefore, the purification and full-characterization of HNL from *Eriobotrya japonica* (*Ej*HNL) were carried out, not only for basic studies of the enzyme, but also for the development of new methods for the enzymatic synthesis of chiral cyanohydrins.

Seeds of *Eriobotrya japonica* were extracted in buffer containing polyvinylpyrrolidone for protecting the enzyme from phenolic compounds in the crude plant extract which form a complex by hydrogen bonding with peptide bond oxygen, or by covalent modification of amino acid residues causing inactivation of the enzyme (Gegenheimer, 1990). The purified *Ej*HNL had a monomer of single subunit. Although the chemical identification was not done, the enzyme was considered to be a glycoprotein, since the enzyme was absorbed to Concanavalin A Sepharose 4B column which binds specifically to the molecules containing sugar residues. Moreover, a broad single band of purified enzyme was observed in SDS-PAGE indicating that the enzyme is a typical glycoprotein. Similar thick bands of glycoprotein on SDS-PAGE were reported in *N*-glycanase from rice seeds (Chang *et al.*, 2000), α-glucosidase from *Schizosaccharomyces pombe* (Okuyama *et al.*, 2005), and glycoprotein produced by *Phoma tracheiphila* (Fogliano *et al.*, 1998). The glycoprotein HNL was observed in *Prunus dulcis*, *Prunus laurocerasus*, *Prunus serotina*, *Prunus lyonii*, *Sorghum bicolor*, and *Ximenia Americana* (Xu *et al.*, 1986; Kuroki and Conn, 1989; Hickel *et al.*, 1996; Yemm and Poulton, 1986; Jansen *et al.*, 1992), while non-glycosylated HNLs were found in *Hevea brasiliensis, Manihot esculenta, Linum usitatissimum,* and *Phlebodium aureum* (Wajant *et al.*, 1995; Hughes *et al.*, 1994; Hickel *et al.*, 1996). HNLs isolated from Rosaceae (Prunoideae and Maloideae subfamilies) are

glycoproteins with molecular mass of 50-80 kDa (Wajant and Effenberger, 1996). Furthermore, several isozymes of HNL from *Prunus* species were isolated, differing slightly in molecular mass, isoelectric point, or carbohydrate side chains, etc (Yemm and Poulton, 1986). *Prunus dulcis* containing three or four isozymes, *Prunus laurocerasus* containing three isozymes, and *Prunus serotina* containing five isozymes were reported (Hickel *et al.,* 1996). In this study, a single purified isozyme of *Ej*HNL was observed according to the following criteria on the purified enzyme. The results from column chromatography showed a single symmetrical peak of purified enzyme. Single values of molecular mass, optimum pH, optimum temperature, and N-terminal amino acid sequence were observed.

The absorption spectra and N-terminal sequence of the purified *Ej*HNL were similar to the FAD-containing HNLs. Moreover, the present work in our group in cloning of gene encoding to *Ej*HNL, we found a conserved FAD-binding region in the gene encoding the enzyme (data not shown). Therefore, *Ej*HNL is most likely a FAD-containing HNL. The unique characteristic of HNLs isolated from the Prunoideae and Maloideae subfamilies of Rosaceae is the presence of FAD. A previous study has shown that the FAD is bound near the active site (Jorns, 1979). This prosthetic group does not play a role in redox reactions but essential for catalysis and provides structural stability to the enzyme. Removal of FAD causes inactivation of the enzyme (Cheng and Poulton, 1993; Seely *et al.,* 1966; Jorns, 1980).

The Michaelis-Menten constant (K_m) of *Ej*HNL for mandelonitrile was 161 μM. A lower K_m value characterizes a higher affinity between substrate and enzyme. HNLs from *Prunus serotina* (K_m of 172 μM), *Prunus dulcis* (K_m of 290 μM), and *Sorghum bicolor* (K_m of 790 μM) exhibited higher K_m values than *Ej*HNL, while a K_m of 93 μM was observed in *Prunus lyonii* (Xu *et al.,* 1986; Yemm and Poulton, 1986).

The optimum pH of *Ej*HNL using the natural substrate mandelonitrile was found to be approximately 5.5. The optimum pH may vary slightly for different substrates or conditions. The optimum pH of FAD-containing HNLs for the decomposition of mandelonitrile was in the range of 5.0-7.0 (Xu *et al.,* 1986; Hickel *et al.,* 1996; Yemm and Poulton, 1986). A non-FAD-containing HNL (*Phlebodium aureum* and *Ximenia americana* HNL) catalyzing the same reaction also showed optimum pH of 5.0-6.5 (Wajant *et al.,* 1995; Kuroki and Conn, 1989), while the optimum pH values of *Linum usitatissimum, Manihot esculenta* and *Hevea brasiliensis* HNLs

were around pH 5.0-6.0 with acetone cyanohydrin as a substrate (Xu *et al.,* 1986; Hughes *et al.,* 1994; Hickel *et al.,* 1996). The optimum temperature of the purified *Ej*HNL was 40 °C while the HNL obtained from *Phlebodium aureum* had an optimum temperature of 35-40 °C on the same substrate (Wajant *et al.,* 1995). It was difficult to determine the exact optimum pH and temperature of HNL from the decomposition of the substrate because of the effect of the non-enzymatic dissociation of the substrate at higher pH and temperature. Excellent stability of *Ej*HNL over a wide range of pH (pH 3-9) and temperature (0-60 °C) was observed. HNLs from *Prunus dulcis* and *Sorghum bicolor* were reported to be stable over a wide pH range (Jansen *et al.,* 1992), while HNL from *Linum usitatissimum* showed stability at pH 6-11, but was less stable under acidic conditions (Albrecht *et al.,* 1993). HNL from *Hevea brasiliensis* was stable for several hours at 30 °C, but above 70 °C was inactivated rapidly (Bauer *et al.,* 1998). HNL from *Sorghum bicolor* showed good stability against heat inactivation; the activity remaining after incubation (60 °C, 60 min) was higher than 60%, and complete inactivation was observed at 70 °C (30 min) (Jansen *et al.,* 1992).

The metal ions were not required for the enzyme activity. The enzymatic activity was inhibited by some heavy metal ions that may chelate enzyme via peptide bond causing precipitation and inactivation of the enzyme. Similar results regarding metal ions were obtained for HNLs from *Ximenia americana*, *Phlebodium aureum*, *Sorghum bicolor,* and *Prunus serotina* (Wajant *et al.,* 1995; Kuroki and Conn, 1989; Bove and Conn, 1961). The cysteine, serine and histidine amino acid residues might be located near the active site and involved in the catalysis. In a similar HNL from *Prunus amygdalus*, an FAD containing HNL, the identified substrate binding site and catalytic site of the enzyme consisted of His-497, Ser-496, Tyr-457, and Cys-328. They formed the hydrogen bonding interactions with the hydroxyl group of the substrate. His-497 also acts as a protonating or deprotonating residue to the carbonyl compound and HCN in the catalysis reaction (Dreveny *et al.,* 2002). Based on the inhibition experiment, it is likely that these residues are also located around the active site of *Ej*HNL. However, the occurrence of these residues around the active site of *Ej*HNL will be made clear by our future primary structure elucidation.

The substrate specificity of *Ej*HNL for the synthesis of chiral cyanohydrins was investigated by measuring the initial velocity of the enzymatic reaction toward the selected aldehydes and the configuration of the chiral cyanohydrins was assigned according to Cahn-

Ingold-Prelog (Eliel *et al.*, 1994). Previous studies on chiral cyanohydrins synthesis focused only on enantiomeric excess (*e.e.*) and the conversion of products, neglecting to measure the initial velocity of the reaction which is the basis of enzymology (Klempier and Griengl, 1993; Griengl *et al.*, 1997; Schmidt, 1996). Even though several two phase systems are used to produce chiral cyanohydrins, a buffer system was employed in this study to avoid the effect of organic solvents on the stability and activity of the enzyme and effect of the partitioning of substrates between organic and aqueous phases. All aldehydes were dissolved in DMSO to increase the solubility of substrates in buffer and KCN was used as a source of cyanide to avoid working with highly toxic HCN. The reaction was performed at pH 4.0, at which the chemical addition of cyanide to aldehydes was mostly suppressed. *Ej*HNL catalyzed the synthesis of cyanohydrins from both aliphatic and aromatic aldehydes. It could be inferred that *Ej*HNL preferred aromatic to aliphatic substrates suggesting the active site of the enzyme to be located near the hydrophobic region and greater specificity for small substrates than bulky substrates. These results showed the potential of using *Ej*HNL for the synthesis of cyanohydrins. The enzyme characterized here can be categorized as a mandelonitrile lyase (EC 4.1.2.10) because it is a (*R*)-HNL active toward mandelonitrile and its N-terminal sequence was similar to that of mandelonitrile lyases reported in this group. HNLs from *Prunus dulcis*, *Prunus mume*, *Manihot esculenta* and *Hevea brasiliensis* showed broad specificity for aliphatic and aromatic substrates (Sharma *et al.*, 2005; Nanda *et al.*, 2005) while HNL from *Linum usitatissimum* had a narrow range of substrates (Albrecht *et al.*, 1993). HNL from *Ximenia americana* showed fairly good specificity toward aromatic aldehydes (Kuroki and Conn, 1989). The specificity to accept more bulky substrates might be improved by genetic engineering. The (*S*)-HNLs from *Hevea brasiliensis* and *Manihot esculenta* have been engineered by substituting bulky amino acids which constrict the entrance of active sites with less bulky amino acids, leading to changes in the specificity of the enzyme to accept larger substrates (Sharma *et al.*, 2005). The enantiomeric excess (*e.e.*) of chiral cyanohydrins synthesized by *Ej*HNL could be further improved by optimizing the reaction conditions to minimize non-enzymatic reactions in organic media. It is of interest to know the entire structure of *Ej*HNL and engineer an enzyme suitable for the synthesis of cyanohydrins.

4.6 Conclusion

A novel hydroxynitrile lyase, *Ej*HNL, was purified to homogeneity and characterized. The *Ej*HNL is a monomer of flavoprotein linked with carbohydrate side chain with a native molecular mass of 72 kDa. The enzyme showed the great stability with regard to pH and temperature. The amino acid residues His, Ser, and Cys might be involved in the catalysis of the enzyme. The enzyme was active toward aromatic and aliphatic aldehydes, therefore the *Ej*HNL is of interest for further applications in cyanohydrin synthesis.

CHAPTER 5

Parameters Influencing Asymmetric Synthesis of (*R*)-Mandelonitrile by A Novel (*R*)-Hydroxynitrile Lyase from *Eriobotrya japonica*

5.1 Abstract

(*R*)-Mandelonitrile was sucessfully synthesized by an enzymatic transcyanation reaction of benzaldehyde and acetone cyanohydrin catalyzed by a hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) in an aqueous-organic biphasic system. Both pH and temperature had a profound effect on the initial velocity and enantiomeric excess (*e.e.*) of the product, (*R*)-mandelonitrile. High enantiomeric purity of the product was observed at low pH and temperature. The optimum pH and temperature to obtain high *e.e.* were pH 4.0 and 10 °C, respectively. The *Ej*HNL was very stable in ethyl acetate, diethyl ether, methyl-*t*-butyl ether, diisopropyl ether, dibutyl ether, and hexane for 12 h. The best solvent for the highest initial velocity and *e.e.* was diethyl ether with an optimum aqueous phase content of 50% (v/v). Under the optimized conditions, the conversion and *e.e.* of (*R*)-mandelonitrile for 3 h were 40 and 99%, respectively. The aqueous phase containing the enzyme also showed considerably efficient reusability for 4 batch reactions.

5.2 Introduction

Recently, the interest of researchers in the synthesis and application of chiral cyanohydrins has markedly increased. The enantiomeric purity of chiral cyanohydrins has become an important criterion in the synthesis of valuable structural moieties including α-hydroxy-aldehyde, vicinal diols, β-amino alcohols, and β-hydroxy-α-amino acids which are building blocks for industrial products such as pharmaceuticals, veterinary products, crop-protecting agents, vitamins and food additives, etc (Wajant and Effenberger, 1996).

The asymmetric synthesis of chiral cyanohydrins has successfully employed hydroxynitrile lyases (HNLs) as the key enzyme. HNLs (EC 4.1.2.10; EC 4.1.2.11; EC 4.1.2.37; EC 4.1.2.39) are classified based on their enantioselectivity into 2 groups. (*R*)-HNLs catalyze the nucleophilic addition of HCN to aldehydes or ketones yielding (*R*)-cyanohydrins, while (*S*)-HNLs catalyze the formation of (*S*)-cyanohydrins (Fechter and Griengl, 2004; Johnson *et al.*, 2000). Nevertheless, asymmetric synthesis of chiral cyanohydrins employing HNLs is influenced by many factors. To achieve the high enantiomeric purity of chiral cyanohydrins, several strategies have been used to suppress the undesired simultaneous non-enzymatic formation of racemic cyanohydrins. The reaction carried out in the aqueous process has been optimized based on the pH and temperature of the reaction (Griengl *et al.*, 1997). Although methods employing water-miscible solvents to produce chiral cyanohydrins have been of much interest, but the non-enzymatic reaction is still a problem (Wehtje *et al.*, 1988). Therefore, biphasic systems with buffer and water-immiscible organic solvents have been developed to minimize the non-enzymatic reaction (Gerrits *et al.*, 2001).

Among the HNLs discovered up to date, only few of HNLs were purified and characterized (Hickel *et al.*, 1996). Moreover, the asymmetric synthesis of cyanohydrins in biphasic systems have been studied with a few HNLs from *Manihot esculenta* (*Me*HNL), *Hevea brasiliensis* (*Hb*HNL), and *Prunus amygdalus* (*Pa*HNL) and differences in some characteristics in each enzyme were observed (Bauer *et al.*, 1999; Costes *et al.*, 1999; Loos *et al.*, 1995; Hickel *et al.*, 2001; Persson *et al.*, 2002). The activity and enantioselectivity of the enzymes in biphasic systems were influenced by many parameters such as pH, temperature, organic solvent, aqueous phase content, and source of enzyme, etc (Watanabe *et al.*, 2004; Castro and Knubovets, 2003;

Wehtje *et al.,* 1997). However, only few paper describing on the biological characteristics of HNL, the unit of HNL used in cyanohydrin syntheses, and the actual initial reaction velocity have appeared in the literature leading to the lack of understanding of the enzyme characteristics and difficult to repeat the experiment. Therefore, the biological characteristics of the HNLs should be characterized to fully utilize the enzymes in their applications and the actual unit of enzyme should be described in the paper.

Recently, a novel (*R*)-hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) (EC 4.1.2.10) was discovered, purified, and characterized by our group. *Ej*HNL showed promising ability in the synthesis of several cyanohydrins in an aqueous system (Asano *et al.,* 2005; Ueatrongchit *et al.,* 2008). Although the synthesis of cyanohydrins by HNL from loquat (*Eriobotrya L.*) was performed in a biphasic system and under micro-aqueous conditions by Lin *et al.* (1999), but there has no report regarding the actual unit of *Ej*HNL used in cyanohydrin synthesis and the characteristics of the enzyme in biphasic systems have not been studied and described elsewhere. Therefore, the vigilant characterization of *Ej*HNL on the synthesis of cyanohydrins in biphasic systems was of much interest for further application of the enzyme.

In this paper, the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL in biphasic system was investigated for the first time. Transcyanation of benzaldehyde and acetone cyanohydrin was employed in this study (Figure 40). Several important parameters influencing the biological characteristic of the *Ej*HNL including pH, temperature, organic solvents, aqueous phase content, substrate concentration, and the enzyme concentration were studied and described. Moreover, all of these parameters were optimized to achieve high initial reaction velocity and enantiomeric purity of the products. The aqueous phase containing the enzyme was found to be reusable under the optimized conditions.

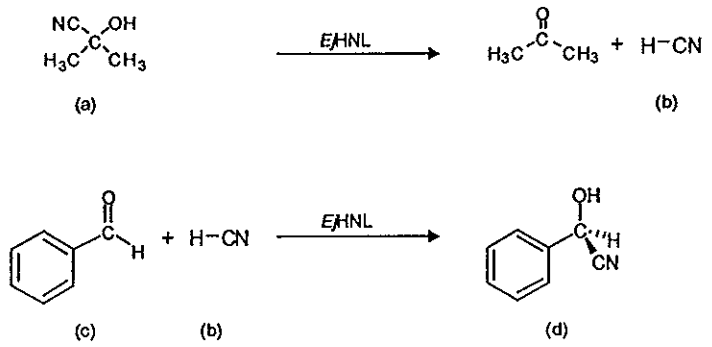


Figure 40. The synthesis of (*R*)-mandelonitrile by transcyanation of acetone cyanohydrin and benzaldehyde using *Ej*HNL. Acetone cyanohydrin (a) was cleaved and released hydrocyanic acid (b) by the enzymatic reaction of *Ej*HNL. Then, the *Ej*HNL catalyze the asymmetric addition of hydrocyanic acid to benzaldehyde (c) yielding the (*R*)-mandelonitrile (d).

5.3 Materails and Methods

5.3.1 Materials and chemicals

Seeds of *Eriobotrya japonica* were purchased from the National Federation of Agricultural Co-operative Associations (Nagasaki, Japan) and stored at 4 °C. All chemicals utilized in the experiments were purchased from commercial sources and used without further purification. Benzaldehyde (redistilled, 99.5+%; Sigma-Aldrich, Inc., USA) and acetone cyanohydrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were also used. The chiral HPLC analysis was performed using a CHIRALCEL OJ-H column (Diacel Chemical Industries Ltd., Osaka, Japan) with a SPD-10A VP UV-vis detector (Shimadzu, Kyoto, Japan) at 254 nm. The eluting solvent was the mixture of *n*-hexane (85%) and isopropanol (15%).

5.3.2. Crude enzyme preparation

Seeds of *Eriobotrya japonica* were sterilized by 0.1% (v/v) sodium hypochlorite and rinsed with de-ionized water. Sterilized seeds were homogenized with 3% polyvinylpyrolidone in 10 mM potassium phosphate buffer, pH 6.0 (100 ml/100 g fresh seed) in a

SMT Process Homogenizer PH91 (SMT company, Tokyo, Japan) at 4 °C. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 28,000 *g*, 4 °C for 30 min. The supernatant was precipitated by 30-80% saturation of $(NH_4)_2SO_4$, and the precipitated enzyme was collected by centrifugation at 28,000 *g*, 4 °C for 30 min. The precipitate was dissolved and dialyzed against the same buffer for 12 h. The enzyme solution was lyophilized. The lyophilized powder (2,270 units/g powder) was used as a crude enzyme.

5.3.3 Activity assay

A reaction mixture in a total volume of 1.0 ml was prepared in a micro-tube. Bezaldehyde (1.0 M in DMSO, 40 µl) was added to sodium citrate buffer (400 mM, pH 4.0), followed by the enzyme solution and a KCN solution (1.0 M, 100 µl). The initial velocity of the reaction was monitored by taking a small aliquot of the reaction mixture (100 µl) and the reaction was stopped by extracting with 700 µl of organic solvent (85% *n*-hexane and 15% isopropanol by volume). The mandelonitrile formed was extracted into the organic layer, centrifuged at 15,000 g for 1 min at 4 °C, and analyzed by HPLC with a CHIRALCEL OJ-H column at 254 nm using a mobile phase of solvent (85% *n*-hexane and 15% isopropanol by volume) at a flow rate of 1.0 ml/min. The retention times of benzaldehyde and (*R*)- and (*S*)-mandelonitrile were about 4.9, 10.2, and 12.7 min, respectively. The reaction progressed linearly in the first five minutes was used for calculating activity.

One unit of HNL activity was defined as the amount of enzyme that produced 1 µmol of optically active mandelonitrile from benzaldehyde per min under standard assay conditions.

5.3.4 Transcyanation reaction in biphasic system

The reaction (total volume of 1.5 ml) was performed in a 2.0 ml micro-tube. Organic solvent containing benzaldehyde was mixed with citrate-phosphate buffer (400 mM) containing the crude enzyme powder. The reaction was initiated by the addition of acetone cyanohydrin, and the mixture was shaken at 1,500 rpm in an incubator shaker (BioShaker M-BR-

022UP, Taitec Corporation, Tokyo, Japan). Aliquots of sample (50 µl) were withdrawn from the organic phase at different time intervals, and mixed with the HPLC solvent (100 µl; *n*-hexane:2-propanol, 85:15). The initial velocity, conversion and enantiomeric excess (*e.e.*) of (*R*)-mandelonitrile formed were analyzed by chiral HPLC. The initial velocity and conversion were calculated using a standard curve of (*R*)-mandelonitrile, while *e.e.* was determined by calculation of the peak areas of the two enantiomers using Eq. (1):

$$\% \text{ Enantiomeric excess } (e.e.) = [R\text{-}S]/[R+S] \times 100 \qquad (1)$$

where *R* and *S* represent the concentrations of the (*R*)-mandelonitrile and (*S*)-mandelonitrile, respectively.

Details of the pH, temperature, benzaldehyde and acetone cyanohydrin concentrations, organic solvent, volume of the aqueous buffer, and enzyme concentration were specific for each case and described in the figure legends.

5.3.5 Influence of organic solvents on enzyme stability

The organic solvents were mixed with 400 mM citrate-phosphate buffer, pH 4.0 (ratio 1:1 by volume) and equilibrated with shaking at 1,500 rpm, 10 °C for 60 min in the incubator shaker (BioShaker M-BR-022UP, Taitec Corporation, Tokyo, Japan). The solution of *Ej*HNL (50 µl, 5 units) was injected into the aqueous phase and mixed gently so as not to disturb the interface. Then, the enzyme activity at zero time was determined with the saturated solvent in the aqueous phase. After that, the mixtures of enzyme solution and organic solvent were shaken to obtain the emulsion between the two phases at 1,500 rpm and 10 °C for 12 h. The mixture was centrifuged at 15,000 g for 1 min at 4 °C to separate the aqueous and organic phase, and the aqueous phase was then withdrawn to assay the remaining activity of the enzyme.

5.3.6 Determination of the partition coefficients for benzaldehyde and mandelonitrile

The partition coefficient for benzaldehyde between the buffer and various organic solvents was determined. Benzaldehyde (20 mM) was dissolved in the organic solvent (500 µl), then mixed with 400 mM citrate-phosphate buffer, pH 4.0 (500 µl). The mixture was equilibrated by shaking at 10 °C for 1 h. The benzalehyde concentration was determined by HPLC with a CHIRALCEL OJ-H column and the partition coefficient was calculated using Eq. (2):

$$\text{Partition coefficient } (K) = C_{org}/C_{aq} \qquad (2)$$

where C_{org} and C_{aq} represent the concentrations of benzaldehyde (g/l) in organic phase and in aqueous phase, respectively.

5.3.7 Reusability

To test the stability of the enzyme in the aqueous phase in repeated use, a batch transcyanation reaction of benzaldehyde (200 mM) and acetone cyanohydrin (400 mM) in a biphasic system of 400 mM citrate-phosphate buffer, pH 4.0 (5 ml) and diethyl ether (5 ml) was conducted by addition of the *Ej*HNL powder (30 units) at 10 °C for 3 h. Then, the aqueous phase containing the enzyme was recovered and dialyzed against the 400 mM citrate-phosphate buffer, pH 4.0 and reused for the next batch reaction under the same conditions.

5.4 Results

5.4.1 Effects of pH and temperature

The effect of pH on the asymmetric synthesis of (*R*)-mandelonitrile from benzaldehyde and acetone cyanohydrin by *Ej*HNL in the biphasic system of diisopropylether (DIPE) and buffer was examined in the range of pH 3-7. The pH of the buffer influenced the

reaction velocity and *e.e.* of the product significantly. The highest initial velocity of *Ej*HNL in the biphasic system was at pH 5 (Figure 41a) and it lost activity at a pH lower and higher than this optimum. The highest *e.e.* (98%) was observed between pH 3.0 and 4.0, while an increase of pH from 4.5 to 7.0 caused a drastic loss in the *e.e.* of the product. At low pH, the spontaneous release of cyanide ion from acetone cyanohydrin was slow leading to the low initial velocity of the enzyme (Kiljunen and Kanerva, 1996). On the other hand, the low pH might suppress the non-enzymatic reaction leading to a high *e.e.* At higher pH, the acceleration of acetone cyanohydrin decomposition occurred providing the low *e.e.* of the product (Griengl *et al.*, 1997).

The optimum conditions to obtain a high *e.e.* of the product were reported to be pH 5.0-5.5 and a temperature of between -5 and 4 °C (Sharma *et al.*, 2005). The effects of pH and temperature on *e.e.* were studied. The higher *e.e.* was obtained at pH 4.0 than 5.5 at the same temperature because the non-enzymatic reaction was almost suppressed (data not shown). Therefore, the effect of temperature was studied at pH 4.0 to minimize the non-enzymatic reaction.

The effect of temperature in the range of 4-50 °C was investigated in the biphasic system of DIPE and citrate-phosphate buffer (pH 4.0). As demonstrated in Figure 41b, the enantioselectivity of *Ej*HNL was certainly influenced by reaction temperature. The initial velocity increased as the temperature increased from 4 to 40 °C, followed by a decrease at higher temperature. When the reaction temperature increased, the chance of a collision between the enzyme and both substrates also increased. This might explain why the formation of enzyme-substrate complexes was enhanced and the reaction rate was improved. Although the highest initial velocity of the reaction was observed at 40 °C, the highest *e.e.* (>99%) was obtained at low temperature, 4-10 °C. The *e.e.* value decreased with an increase of temperature due to an acceleration of the non-enzymatic reaction at high temperature. High temperature might increase the possibility of non-enzymatic collision between molecules of benzaldehyde and HCN released from acetone cyanohydrin and cause the racemization of the product.

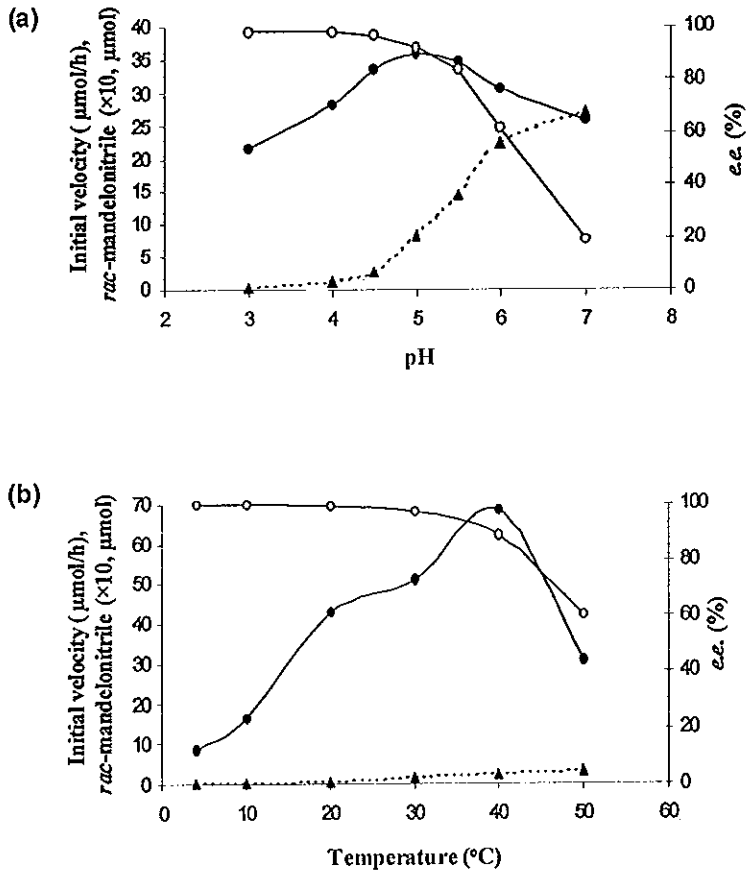


Figure 41. Effect of pH (a) and temperature (b) on transcyanation catalyzed by *Ej*HNL. Initial velocity (●), *e.e.* (○), and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer and DIPE, containing 5 U of *Ej*HNL. The reaction was performed at 30 °C for pH effect and pH 4.0 for temperature effect.

5.4.2 Effect of organic solvents

Organic solvent is essential to activity and enantioselectivity of enzymes. It has a large influence on enzyme enantioselectivity, but in some cases it was found to have no effect (Ke and Klibanov, 1998; Wolff *et al.*, 1997). Ethyl acetate (EA; log *P* 0.67), diethyl ether (DEE; log *P* 0.85), methyl-*t*-butyl ether (MTBE; log *P* 1.4), diisopropyl ether (DIPE; log *P* 1.9), dibutyl ether (DBE; log *P* 2.9) and hexane (HEX; log *P* 3.5) were used as the organic phase in biphasic system. The initial velocity of the reaction was significantly affected by the organic solvents. The

best solvent giving the highest initial velocity was DEE, while a decrease in the initial velocity of the reaction was observed with solvents having a log *P* value higher and lower than 0.85 (Figure 42).

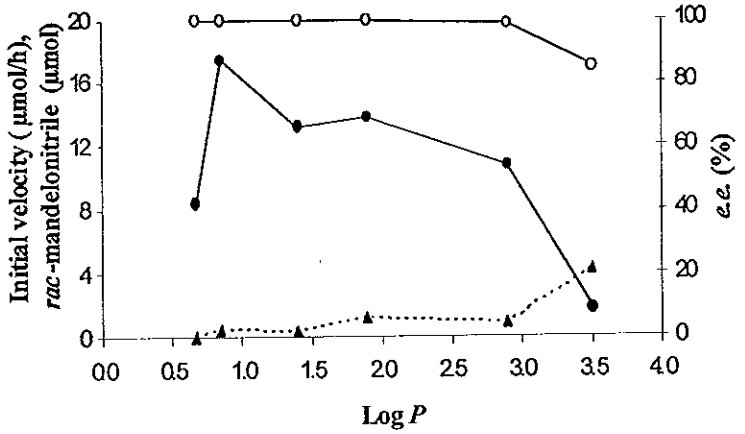


Figure 42. Effect of organic solvent on transcyanation reaction catalyzed by *Ej*HNL. Initial velocity (●), *e.e.* (○), and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer, pH 4.0, 10 °C, containing 5 U of *Ej*HNL with various organic solvents.

*Ej*HNL showed weak activity in the EA and HEX biphasic systems. Good stability of *Ej*HNL in all biphasic systems was observed and the stability was not correlated with the log *P* of the organic solvent as shown in Figure 43a. The stability of *Ej*HNL after incubation with various organic solvents was determined and compared to that of initial activity in aqueous phase saturated with the organic solvent. The remaining activity of the enzyme was more than 80% in EA, DBE, DEE, DIPE, MTBE and HEX after incubation for 12 h. Log *P* values between 0.67 and 2.9 had no significant effect on the *e.e.* value, while a decrease in *e.e.* was observed at a log *P* of 3.5 for HEX.

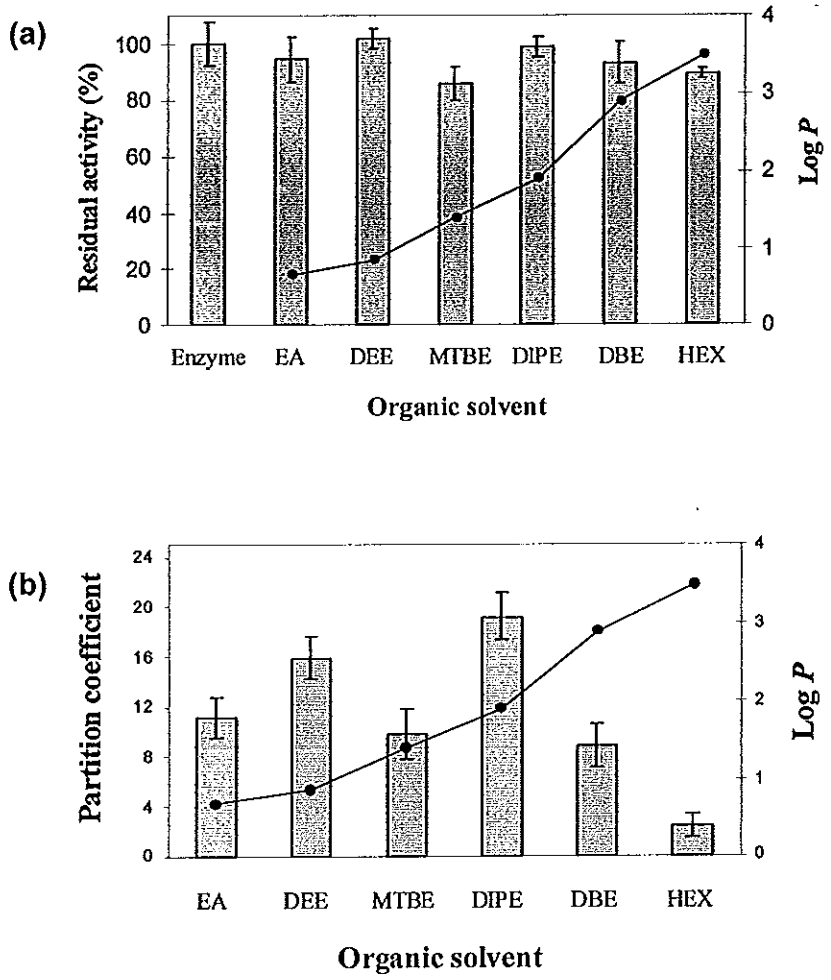


Figure 43. Effect of organic solvents on stability of *Ej*HNL (a) and partition coefficient of mandelonitrile (b). (▨) Residual activity of *Ej*HNL after incubated for 12 h or partition coefficient of mandelonitrile, (●) Log *P* of organic solvent.

The partition coefficient for benzaldehyde in biphasic systems was determined (Figure 43b). The partition coefficient for benzaldehyde in the organic phase should be high to minimize the inhibition of enzyme in the aqueous phase and reduce the non-enzymatic reaction rate. DIPE and DEE were suitable solvents for the partitioning of benzaldehyde to the organic phase, while EA, MTBE and DBE showed moderate values for the partition coefficient. In the case of HEX, the partition of benzaldehyde was very low, therefore benzaldehyde almost dissolved in the aqueous phase with acetone cyanohydrin. This might increase the non-enzymatic reaction and cause the lower *e.e.* of the product.

5.4.3 Effect of aqueous phase content

The aqueous phase content is an important factor in the use of an enzyme in an organic solvent. The effect of aqueous phase content on the enzyme reaction was studied at 5-60% (v/v) with the volume of organic phase fixed at 1 ml. A content of less than 5% (v/v) caused aggregation of the enzyme.

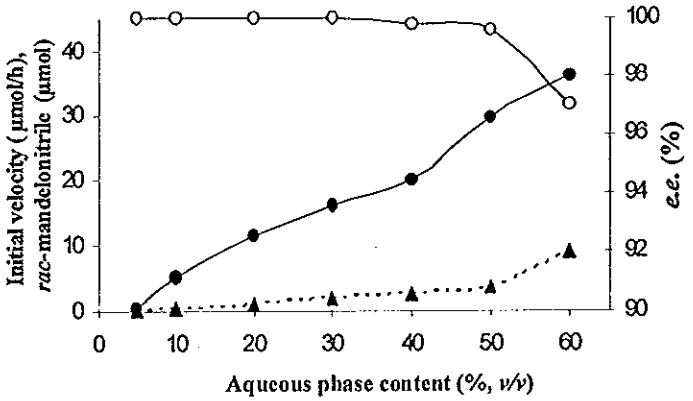


Figure 44. Effect of aqueous phase content on transcyanation reaction catalyzed by *Ej*HNL. Initial velocity (●), *e.e.* (○), and non-enzymatic reaction (▲) in biphasic system of DEE (1 ml) and buffer (pH 4.0, vary volume of aqueous phase content) at 10 °C containing benzaldehyde (250 mM), acetone cyanohydrin (500 mM), and 5 U of *Ej*HNL.

The aqueous phase content affected the reaction rate more clearly than the *e.e.* (Figure 44). Increasing the aqueous phase content in biphasic system caused the initial reaction rate to rise linearly. The high water content might provide more flexibility to the enzyme molecules (Klibanov, 1997), increase the mass transfer of the enzyme and substrates (Willeman *et al.*, 2002), and reduce the chance of contact with the interface of the biphasic reaction which might inactivate the enzyme (Hickel *et al.*, 1998). Furthermore, the high aqueous phase content stimulated the decomposition of acetone cyanohydrins to HCN (Kiljunen and Kanerva, 1996), yielding the corresponding high reaction rate.

In our study, there was no straightforward correlation between *e.e.* and the initial velocity of the reaction. A high *e.e.* (≥99%) was obtained at aqueous phase content of 5-50%

(v/v). On the other hand, an excess of aqueous phase (more than 50%) accelerated the spontaneous non-enzymatic reaction and led to a fall in the *e.e.* of the product.

5.4.4 Effect of substrate concentration

The effect of benzaldehyde and acetone cyanohydrin on the initial reaction velocity and enantiomeric purity of (*R*)-mandelonitrile was studied. The benzaldehyde concentration was varied in the range of 10-300 mM, while keeping the acetone cyanohydrin concentration at 500 mM. The initial reaction velocity of *Ej*HNL increased linearly when the benzaldehyde concentration increased up to 200 mM and a constant initial velocity was observed at 200-300 mM (Figure 45a). The effect of the acetone cyanohydrin concentration was investigated while the benzaldehyde concentration was fixed at 200 mM. The increase in the initial reaction velocity of *Ej*HNL was linearly correlated with the acetone cyanohydrin concentration (Figure 45b). The result indicated that the enzyme was not inactivated by a high concentration of acetone cyanohydrin. In contrast, increasing the benzaldehyde or acetone cyanohydrin concentration caused a slight decrease in the *e.e.* of the product due to an enhanced spontaneous chemical reaction between substrates. However, an *e.e.* of more than 98% was observed in all cases. KCN was not suitable as a source of cyanide because the non-enzymatic reaction was occurred rapidly yielding mandelonitrile with a significantly low *e.e.* (data not shown).

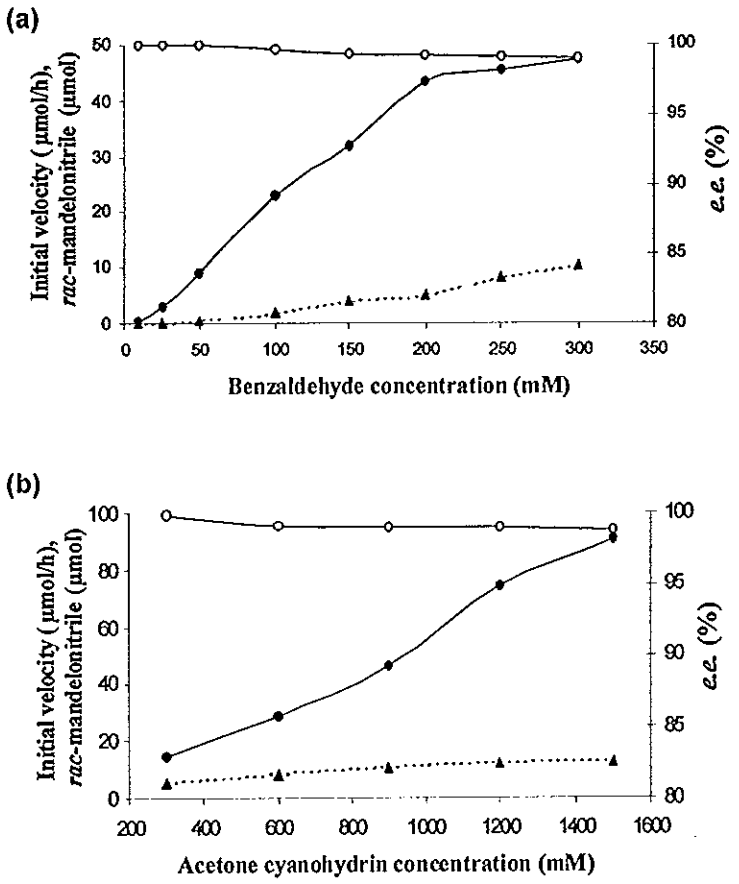


Figure 45. Effect of benzaldehyde (a) and acetone cyanohydrin (b) concentration on transcyanation reaction catalyzed by *Ej*HNL. Initial velocity (●), *e.e.* (○), and non-enzymatic reaction (▲) in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE at 10 °C. To determine effect of benzaldehyde, concentration of acetone cyanohydrin was kept constantly at 500 mM, while concentration of benzaldehyde was kept at 200 mM when studied on effect of acetone cyanohydrin. The 5 Units of *Ej*HNL was used in all cases.

5.4.5 Effect of enzyme concentration

A linear increase in the initial velocity with an increase in enzyme concentration up to the optimum of 13.3 units/ml reaction mixture was observed (Figure 46). The increase of enzyme concentration in the biphasic reaction might cause the mass transfer rate to rise (Willeman *et al.*, 2002) and might be involved in the raising of initial velocity. The excess amount of enzyme (more than 13.3 units/ml reaction) caused a decrease in initial velocity that might be due to the aggregation of the enzyme powder. In this case, the viscosity and turbidity of the enzyme solution were observed when the enzyme was added at a concentration beyond the optimum. The aggregation might limit the mass transfer between the enzyme and substrates. A slight drop in the *e.e.* of the product was found when the amount of the enzyme was increased.

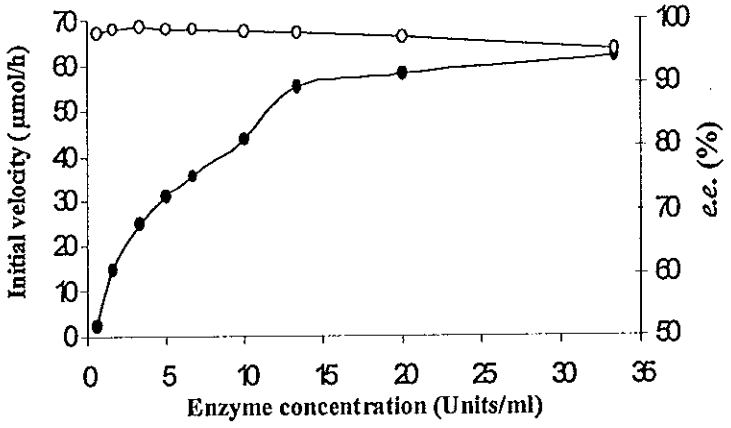


Figure 46. Effect of enzyme concentration on transcyanation reaction catalyzed by *Ej*HNL. Initial velocity (●) and *e.e.* (○). The reaction was performed in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE, at 10 °C containing benzaldehyde (200 mM), acetone cyanohydrin (400 mM), and various concentration of *Ej*HNL.

5.4.6 Synthesis of (*R*)-mandelonitrile and reusability of *Ej*HNL

The synthesis of (*R*)-mandelonitrile by *Ej*HNL was demonstrated for the first time. The time-course of (*R*)-mandelonitrile synthesis by the transcyanation reaction is shown in Figure 47a. Linear progress in the reaction was observed in the first hour with the conversion and *e.e.* of (*R*)-mandelonitrile being 40 and 99 %, respectively. A constant *e.e.* value was observed from 3 to 24 h which indicated the total suppression of the non-enzymatic reaction. The transcyanation of (*R*)-mandelonitrile was compared between *Ej*HNL and HNL from *Prunus amygdalus* (*Pa*HNL) as shown in Figure 47b. The time course was not significantly different in *e.e.* (99%) or conversion (35-38%).

The reusability of *Ej*HNL in asymmetric (*R*)-mandelonitrile synthesis employing a biphasic system was studied. The enzyme in aqueous phase was recovered and successfully used for the next reaction. As the results show in Table 11, a residual activity of 90% of the reused *Ej*HNL in aqueous phase remained after four cycles and *e.e.* did not change markedly.

Table 11. (*R*)-mandelonitrile synthesis in repeated batch process by *Ej*HNL.

Batch	Initial velocity (μmol/h)	*e.e.* (%)	Conversion (%)
1	63.7	99.7	40.4
2	62.1	99.4	39.3
3	59.8	98.5	38.1
4	57.3	98.2	36.8

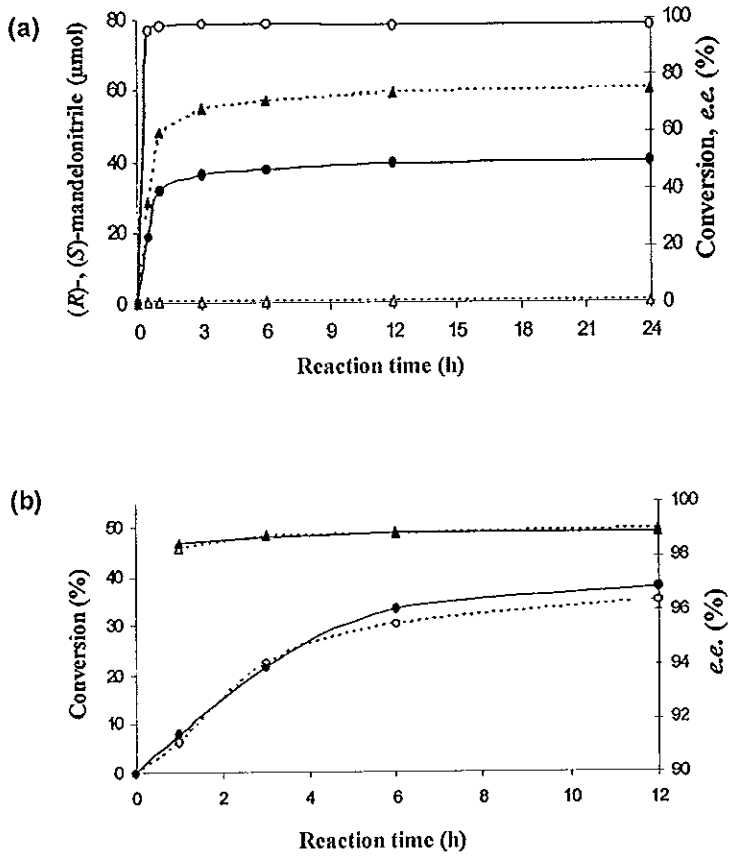


Figure 47. Transcyanation of (*R*)-mandelonitrile synthesis by *Ej*HNL. (a) Time course for transcyanation of (*R*)-mandelonitrile synthesis by *Ej*HNL. The *e.e.* (—○—), conversion(—●—), (*R*)-mandelonitrile (--▲--) and (*S*)-mandelonitrile (--Δ--) was monitored for 24 h. (b) A comparison of transcyanation of (*R*)-mandelonitrile synthesis by *Ej*HNL and *Pa*HNL. The conversion of *Ej*HNL (—●—) and *Pa*HNL (--○--) and the *e.e.* of *Ej*HNL (—▲—) and *Pa*HNL (--Δ--) was monitored for 12 h. The (*R*)-mandelonitrile synthesis reaction was performed in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE (50%, v/v) at 10 °C containing benzaldehyde (200 mM), acetone cyanohydrin (400 mM), and enzyme (13.3 units/ml).

5.5 Discussion

Among the HNLs discovered up to date, a few of these enzymes were purified and characterized. Many reports have dealt with the synthesis of cyanohydrins to obtain the high yield and enantiomeric purity of product. The lack of information on the biological properties of HNLs has limited not only the understanding of the enzyme reaction, but also their applications.

A novel (*R*)-hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) (EC 4.1.2.10) was isolated, purified, and characterized for the first time by our group. *Ej*HNL performed considerably well in the synthesis of various asymmetric cyanohydrins in a buffer system, but the non-enzymatic reaction was still a problem, leading to poor enantiomeric purity of the desired chiral products (Asano *et al.*, 2005; Ueatrongchit *et al.*, 2008). A biphasic system was an interesting way to minimize the amount of undesired product. So far the synthesis of cyanohydrins employing a HNL from loquat (*Eriobotrya L.*) in biphasic systems and single phase organic solvents was reported (Lin *et al.*, 1999), however, there is no literature of the biological characteristics of the *Ej*HNL in the system containing organic solvent. Most of the cyanohydrin synthesis catalyzing by HNLs always employ EA or DIPE as organic phase (Hickel *et al.*, 2001; Lin *et al.*, 1999) since it might be commonly believed that these organic solvents are suitable for the enzymes. Moreover, an excess amount of crude enzyme was added to achieve the high *e.e.* of products without concerning about enzyme activity. Therefore, the objective of this study is to fill the lack information of biological characteristics of *Ej*HNL and optimized the reaction condition to achieve high initial reaction velocity and enantiomeric purity of the products.

We successfully demonstrated the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL in a biphasic system and achieved a higher *e.e.* (99%) of the product for the first time, as compared with the *e.e.* of 81% in the biphasic system reported in a previous work (Lin *et al.*, 1999). Furthermore, several parameters influencing the initial velocity and enantiomeric purity of the product were investigated and optimized. Especially, diethyl ether was found to be the most suitable organic solvent in biphasic system for *Ej*HNL which differ from previous reports of other HNLs (Gerrits *et al.*, 2001; Bauer *et al.*, 1999; Costes *et al.*, 1999; Persson *et al.*, 2002; Lin *et al.*, 1999).

In our study, both pH and temperature absolutely affected the *e.e.* of the product. The pH and temperature (pH 5 and 40 °C, respectively) giving the highest initial velocity of the reaction were not suitable for asymmetric synthesis of the (*R*)-mandelonitrile. A low *e.e.* of the desired product was obtained when using the pH and temperature giving the highest initial velocity. Under these conditions, the non-enzymatic reaction was incited and produced the racemate of mandelonitrile leading to the fall in *e.e.* As demonstrated in Figure 40, a low pH and temperature should be maintained during the reaction to suppress the non-enzymatic reaction and

achieve the highest *e.e.* of the product. These results agreed with previous reports on HNLs from *Hevea brasiliensis*, *Manihot esculenta*, *Sorghum bicolor*, and *Prunus amygdalus* (Costes *et al.*, 1999; Persson *et al.*, 2002). A similar effect of temperature on the enantioselectivity of enzymes was found for lipase and hydrolase which are enantioselective biocatalysts (Lo'pez-Serrano *et al.*, 2001; Sakai, 2004; Wang and Tsai, 2005). Therefore, pH and temperature might be important parameters to control the enantioselectivity of enzymes to achieve a high *e.e.* of their products.

Surprisingly, the initial velocity of *Ej*HNL was significantly affected by the type of organic solvent. *Ej*HNL exhibited the best initial activity in DEE system which differed from previous work on other HNLs which almost performed the enzymatic reaction in biphasic system of DIPE or EA (Hickel *et al.*, 2001; Lin *et al.*, 1999). Therefore, it is necessary to study the characteristics of the enzymes in organic solvent before employing them in the system containing organic solvent, since the conformation of the enzyme might be affected by the change in dielectric properties of the reaction medium caused by introducing an organic solvent, leading to unfolding and a change in activity of the enzyme (Ogino and Ishikawa, 2001). Organic solvents do not only influence the initial velocity of the enzyme, but also affect enzyme stability (Bauer *et al.*, 1999; Hickel *et al.*, 1998). *Ej*HNL was very stable in all biphasic systems used, while HNL from *Prunus amygdalus* showed no lose activity after incubation in DIPE, and MTBE, but unstable in heptane and DBE (Hickel *et al.*, 2001). In our study, the change in log *P* of organic solvent was not correlated with the *e.e.* value of the product, but the partition of benzaldehyde and HCN in the organic and aqueous phases was important to control the enantiomeric purity of the product. For biphasic systems, solvents having no harmful effects on the activity and stability of the enzyme, and a high partition for the aldehyde substrate and cyanohydrin product but a low partition for cyanide, are required. In the case of the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL, DEE was the best solvent, while EA and HEX were not suitable for the enzyme. EA was also reported to be an unsuitable solvent for *Hb*HNL causing strong inhibition of its activity (Bauer *et al.*, 1999), while *Pa*HNL showed the lowest level of activity with MTBE and much higher levels found in a more non-polar solvent than MTBE such as BME (bultyl methyl ether), DIPE, DBE, and heptane (Hickel *et al.*, 2001). Enantioselectivity of *Hb*HNL catalyzing the addition of HCN to 3-phenylpropanal was not affected by log *P* of organic solvent (Persson *et al.*, 2002), whereas that of *Me*HNL catalyzing the transcyanation of acetyltrimethylsilane and acetone

cyanohydrin was, and high hydrophobicity of the organic solvent caused the low *e.e.* of the product (Xu *et al.*, 2004).

A small amount of water is required for the catalytic activity of an enzyme. However, the quantity of water required varies (Lin *et al.*, 1999; Chen *et al.*, 2001; Han *et al.*, 1998; Klibanov, 1989). Actually, the optimum amount of water depends on several parameters, including type of enzyme, polarity of the enzyme active site, type of organic solvent, substrate and reaction conditions. In the case of the transcyanation of (*R*)-mandelonitrile by *Ej*HNL in the biphasic system of DEE and buffer, the optimum amount of water was 50% v/v to obtain high initial velocity of enzyme and *e.e.* of the product. Several papers reported differently on the optimum aqueous content for initial velocity of HNLs in cyanohydrin synthesis. A similar result was observed for the transcyanation of acetyltrimethylsilane by *Me*HNL in which a raise in initial velocity was provided with the increase in aqueous phase content from 13-60% (v/v) (Xu *et al.*, 2004). An optimum water content of 1.0-1.5% (v/v) was reported for *Hb*HNL catalyzing the synthesis of 3-phenylpropionaldehyde cyanohydrin in DBE as the organic phase (Costes *et al.*, 1999). Furthermore, an optimum water content of 0.54% was observed for *Hb*HNL catalyzing the synthesis of the same product in DIPE (Persson *et al.*, 2002).

A difference in the correlation between the optimum water content and *e.e.* of product in cyanohydrin synthesis was reported previously. In the case of *Hb*HNL, the presence of enough water phase to form a biphasic system did not affect the enantioselectivity of the enzyme (Persson *et al.*, 2002), while increasing the aqueous phase led to a decrease of *e.e.* in the transcyanation of acetyltrimethylsilane by *Me*HNL (Xu *et al.*, 2004). Micro-aqueous conditions were also found to suppress the non-enzymatic reaction sufficiently (Lin *et al.*, 1999; Chen *et al.*, 2001; Han *et al.*, 1998). Therefore, it is necessary to optimize the aqueous phase content for cyanohydrin synthesis.

Substrate and enzyme concentrations are parameters influencing the initial velocity of a reaction and enantiomeric purity of the product. In this study, benzaldehyde and acetone cyanohydrin were the substrates. An increase of substrate concentration cause an increase in initial velocity but a slight decrease in *e.e.* due to the increase in the non-enzymatic reaction. Stability of *Ej*HNL at high concentrations of benzaldehyde and acetone cyanohydrin was

observed in this study. However, *Hb*HNL was stable with 3-phenylpropionaldehyde while the high concentration of HCN had a negative influence on the enzyme (Costes *et al.,* 1999).

The previous studies on cyanohydrin synthesis used an excess amount of enzyme to achieve a high *e.e.* without measuring the initial activity and optimizing the amount of enzyme (Lin *et al.,* 1999; Kiljunen and Kanerva, 1996; Hanefeld *et al.,* 2001). In this study, we did optimize the enzyme concentration, where the highest reaction velocity and enantiomeric purity were achieved. The excess amount of enzyme employed in the system caused the decrease in the initial activity. The optimum enzyme concentration should be used for the production of cyanohydrins to achieve the highest level of activity and the lowest cost.

In the present study, we successfully demonstrated the transcyanation of (*R*)-mandelonitrile by a novel *Ej*HNL for the first time. All parameters play an important role in the activity and enantioselectivity of the enzyme, therefore it is necessary to study and optimize all parameters to achieve the highest level of activity and enantiomeric purity of the product. Furthermore, the reusability of the enzyme and ease of product separation show the potential of *Ej*HNL in (*R*)-mandelonitrile synthesis especially on an industrial scale.

5.6 Conclusion

The study on biological characteristics of *Ej*HNL demonstrated that the initial activity and enantioselectivity of *Ej*HNL in the aqueous-organic biphasic system were influenced by several parameters. The organic solvent is a markedly important parameter influencing on the initial reaction velocity. For *Ej*HNL, the suitable organic solvent for (*R*)-mandelonitrile synthesis was DEE that gave the highest initial velocity for *Ej*HNL. The synthesis of (*R*)-mandelonitrile in a biphasic system at low pH and temperature proved to be useful for improving the enantiomeric purity of the product. The highest initial velocity of the reaction and *e.e.* of the product were obtained after optimization of the organic solvent, aqueous phase content, substrate concentration, and enzyme concentration. Furthermore, the possibility of efficient reusing of the enzyme in the aqueous phase makes the enzyme and system attractive for practical applications.

CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

1. Several cyanogenic plants were investigated by screening for the presence of cyanide by picrate paper method. In the same plant family, some plants were cyanogenic plants, while some plants were not. The releasing of cyanides might be influenced by the presence of both hydrolytic enzyme (β-glucosidase) and cyanogenic glycoside collected in plant tissue. The cyanogenic glycoside might be hydrolyzed by β-glucosidase yielding the intermediate cyanohydrins, and then the cyanohydrins might be decomposed spontaneously or catalyzed by HNLs to cyanide and carbonyl compounds. Actually, the cyanohydrin is unstable in neutral to alkaline pH and ambient to high temperature, then the releasing of cyanide from cyanohydrin might occurred spontaneously without HNLs. The presence of HNLs was not observed in every cyanogenic plant, since the presence of HNL might be controlled genetically by the plant genome. The novel HNL from *Passiflora edulis* was observed in leaves, seeds, and rind of the plant and the enzyme showed promising ability in cyanohydrin synthesis.

2. The transcyanation of (*R*)-mandelonitrile synthesis by a novel *Pe*HNL was successfully demonstrated in this present study for the first time. It is necessary to study and optimize all parameter to obtain the high initial velocity of reaction and enantiomeric purity of the chiral product, due to several parameters influenced on the reaction complicatedly. Moreover, the appropriate biphasic system for synthesis the (*R*)-mandelonitrile with high enantiomeric purity in our study showed the reusability of enzyme and easily to recover the product in downstream process.

3. A novel hydroxynitrile lyase, *Ej*HNL, was purified to homogeneity and characterized. The *Ej*HNL is a monomer of flavoprotein linked with carbohydrate side chain with a native molecular mass of 72 kDa. The enzyme showed the great stability with regard to pH, temperature. The amino acid residues His, Ser, and Cys might be involved in the catalysis of the

enzyme. The enzyme was active toward aromatic and aliphatic aldehydes, therefore the *Ef*HNL is of interest for further applications in cyanohydrin synthesis.

4. The study on biological characteristics of *Ef*HNL demonstrated that the initial activity and enantioselectivity of *Ef*HNL in the aqueous-organic biphasic system were influenced by several parameters. The organic solvent is a markedly important parameter influencing on the initial reaction velocity. For *Ef*HNL, the suitable organic solvent for (*R*)-mandelonitrile synthesis was found to be DEE that gave the highest initial velocity for *Ef*HNL. The synthesis of (*R*)-mandelonitrile in a biphasic system at low pH and temperature proved to be useful for improving the enantiomeric purity of the product. The highest initial velocity of the reaction and *e.e.* of the product were obtained after optimization of the organic solvent, aqueous phase content, substrate concentration, and enzyme concentration. Furthermore, the possibility of efficiently reusing of the enzyme in the aqueous phase makes the enzyme and system attractive for practical applications.

6.2 Future works

1. The primary structure of *Ef*HNL and *Pe*HNL should be studied to understand the nature of the enzyme. The HNLs should be cloned and expressed in microorganisms for further production in the industrial scale.

2. The genetic engineering of the *Ef*HNL and *Pe*HNL should be done to increase the possibility to accept wide substrate range, and increase the catalytic activity.

3. The condition of cyanohydrins synthesis should be further studied to improve the yield of cyanohydrins product, and scaled up to further production in the industrial scale.

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