國立交通大學

應用化學系所

博士論文

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Isolation and characterization of a phenol degrading *Candida albicans* TL3 and the study of its Catechol 1,2-dioxygenase

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摘要

本研究已從土壤裡單離出一株可利用酚做為唯一碳源的酵母菌－*Candida albicans* TL3；相較於其它可降解酚之微生物而言，這菌株不僅對酚具有較高的耐受性而且分解酚的速率也相對較快，它對酚的降解能力目前是第一次被發現的。基於酵素活性、層析及質譜等分析，我們推論此菌株是經由 ortho-fission 途徑分解酚。涉及此途徑的相關酵素－phenol hydroxylase 和 catechol 1,2-dioxydanase 兩者皆是可誘導性酵素，當 *C. albicans* TL3 被培養在培養液分別含酚濃度是 22 mM 和 10 mM 時，可達到最大活性。另外，此菌株除了能降解甲醛樹脂工廠廢液中的酚之外，亦可對甲醛加以降解。

藉由硫酸銨沉澱、Sephadex G-75 凝膠過濾和 HiTrap Q Sepharose 管柱層析，可從 *C. albicans* TL3 中分離出一高純度的 catechol 1,2-dioxydanase（1,2-CTD）；此酵素是由相同的兩個單體所組成的，每一個單體的分子量是 32,000 Da 且含一個鐵離子。此真菌的 1,2-CTD 的 p*I* 值、最適溫度和最適酸鹼值分別為 5.3~5.7、25℃和 pH 8.0。由受質特異性之研究顯示，此酵素應屬於 type I catechol 1,2-dioxydanase。這是首次有關來自真核細胞之 catechol 1,2-dioxygenase 的研究

報導。在二維電泳膠片上，可看到此純化的 1,2-CTD 具有五個分子量相近但等電點稍微不同的蛋白質點；這五種異構型態的 1,2-CTD 可能是不同程度的轉譯後修飾作用所造成的。利用 Edman 降解和 MALDI-TOF/TOF 對經胰蛋白酶水解後的此 1,2-CTD 裂解之胜肽片段進行序列分析，所得的序列結果可比對到一與其具高度相同性來自於 *Candida albicans* SC5314 的假想蛋白質－CaO19_12036 (GenBank accession no. XM 717691)；我們建議此一假想蛋白質應該是一 1,2-CTD。

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Abstract

A yeast strain isolated from soil was able to utilize phenol as the sole carbon source and was further identified as *Candida albicans* TL3. This microbe possesses higher tolerance on phenol (24 mM) as well as stronger activity on the rate of phenol degradation than other microorganisms at 30℃. The capability of this strain on phenol degradation is first reported herein. Based on the enzymatic, chromatographic and mass spectrometric analyses, we concluded that *C. albicans* TL3 follows the ortho-fission pathway on phenol degradation. The optimal activity of phenol hydroxylase and catechol 1,2-dioxygenase were found when this strain grew in culture media containing 22 mM and 10 mM phenol, respectively. In addition to phenol, *C. albicans* TL3 also exhibited catalytic power on degrading formaldehyde in wastewater directly obtained from phenolic resin-producing factory.

The catechol 1,2-dioxygenase (1,2-CTD) induced from *Candida albicans* TL3 was purified via ammonium sulfate precipitation, Sephadex G-75 gel filtration and

HiTrap Q Sepharose column chromatography. The enzyme was purified to homogeneity and characterized to be a homodimer, with a molecular weight of 32,000 Da for each subunit. The investigation of this eukaryotic 1,2-CTD revealed that the iron content for each subunit, pI value, optimal temperature, and optimal pH are 1 iron/subunit, 5.3~5.7, 25℃ and pH 8.0, respectively. Substrate analysis showed that the purified enzyme belongs to the type I catechol 1, 2-dioxygenase. The study on this eukaryotic 1,2-CTD was reported for the first time. On 2-D gel analysis of the purified 1,2-CTD, five spots with approximately similar molecular weight but with different p*I*s were found. These spots were further analyzed by MALDI-TOF mass spectrometry. Results suggested that these spots (isotypes) were derived from the same 1,2-CTD. Peptide sequencing on fragments of 1,2-CTD by Edman degradation and MALDI-TOF/TOF analysis provide information of amino acid sequences for BLAST search, the outcome of the BLAST revealed that this eukaryotic 1,2-CTD has high identity with a hypothetical protein, CaO19_12036, from Candida albicans SC5314 (GenBank accession no. XM 717691). We, thus, suggested that the hypothetical protein should be 1,2-CTD.

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Chapter 1

Background

1.1 Introduction

Phenol and phenolic compounds have been a hazardous pollutant in the environment throughout the last century. Phenols compounds are present in many industrial effluents including resins, petrochemical, coal gasification, ceramic, pharmaceutical, and dye manufacturing industry (Swoboda-Colberg 1995). Another toxic compound — formaldehyde, sometimes be found in phenolic pollutant. The toxicity and bactericide effects of phenol often cause problems with the process of wastewater treatment. For example, phenol can be toxic to fish at a concentration of 5 mg/liter and imparts an objectionable taste to drinking water at far lower concentration (Throop 1975/1977). The physical and chemical methods of phenol treatment require many processing steps, which are not only costly and often produce other toxic end products as well (Kobayashi and Rittmann 1982). Biodegradation has been considered as one of the safest, least costly and most socially acceptable methods of decontamination of fouled environment. The remarkable ability of microbes to break down chemicals is useful not only in pollution remediation but also serves as a potential tool in pollutant detection.

Many microorganisms including *Acinetobacter calcoaceticus* (Fewson 1967),

Pseudomonas putida (Bayly and Wigmore 1973), *Alcaligenes eutrophus* (Hughes and Bayly 1983), *Streptomyces setonii* (Antai and Crawford 1983), *Bacillus stearothermophilus* (Gurujeyalakshmi and Oriel 1989), *Rhodopseudomonas palustrisand* (Rahalkar et al. 1993), *Comamonas testosterone* (Yap et al. 1999), *Trichosporon cutaneum* (Neujahr and Varga 1970), *Rhodotorula mucilaginosa* (Cook and Cain 1974), *Candida tropicalis* (Neujahr 1974), *Ochromonas danica* (Semple and Cain 1996), and aquatic fungi (Ristanovic et al. 1975), were found to be capable of degrading phenol at different level of concentration. These microorganisms utilize commonly two different pathways of phenol degradation: ortho- and meta-cleavage pathways (Fig. 1-1). Both pathways use phenol hydroxylase in the first step of degradation but they are different in the second degradation step, eukaryotes and prokaryotes use intradiol dioxygenase (ortho-cleavage) and extradiol dioxygenase (meta-cleavage), respectively (Bastos et al. 2000; El-Sayed et al. 2003).

Phenols initial conversion to catechols by phenol hydroxylase. The aromatic ring of catechols are further opened by intradiol dioxygenase via an ortho-cleavage pathway, to cis, cis-muconate or by extradiol dioxygenase via a meta-cleavage pathway, to 2-hydroxymuconic semialdehyde, respectively, which is subsequently metabolized towards the tricarboxylic acid cycle (Ngai et al. 1990; Chen and Lovell 1990; Aoki et al. 1990). Mostly, both types of catechols dioxygenase use non-heme

iron as cofactor (intradiol dioxygenase and extradiol dioxygenase contain ferric and ferrous form, respectively) and catalyze the addition of both atoms of molecular oxygen to catechols (Nozaki 1979). Intradiol dioxygenases are classified into two structurally different families: catechols 1,2-dioxygenase and protocatechuate 3,4-dioxygenase (3,4-PCD), which are specific for catechol (or its derivatives) and hydroxybenzoates, respectively. In general, catechols 1,2-dioxygenases are dimeric proteins with identical or similar subunits. Catechols 1,2-dioxygenases are classified into two types according to their substrate specificities (Ferraroni et al. 2004): type I enzymes are specific for catechols, alkylated catechols (catechol 1,2-dioxygenases, 1,2-CTDs) (Nakai et al. 1990; Eck et al. 1991; Murakami et al. 1997; Briganti et al. 1997; Shen et al. 2004), and hydroxyquinols (hydroxyquinol 1,2-dioxygenases, 1,2-HQDs) (Latus et al. 1995); type II enzymes are specific for chlorocatechols (chlorocatechol 1,2-dioxygenases, 1,2-ClCTDs) (Broderick et al. 1991; Van der Meer et al. 1993; Maltseva et al. 1994) (Fig. 1-2).

Catechol 1,2-dioxygenases play important roles in the degradation pathways of various aromatic compounds and are ubiquitous in microorganisms (Broderick et al. 1991; Latus et al. 1995; Sauret-Ignazi et a1. 1996; Briganti et al. 1997; Strachan et al. 1998; An et al. 2001; Shen et al. 2004; Ferraroni et al. 2004; Ferraroni et al. 2005). Catechol 1,2-dioxygenases from prokaryotic cells have been extensively characterized

in terms of their biochemical and structural properties and their amino acid sequences (Kim et al. 1997; Eulberg et al. 1997; Feng et al. 1999; Pessione et al. 2001; Kim et al. 2002; Caposio et al. 2002; Kim et al. 2003; Earhart et al. 2005). Various catechol 1,2-dioxygenases isozymes that differ in the composition of their subunits are found in microorganisms such as *Pseudomonas arvilla* C-1, *Acinetobacter lwoffii* K24, *Frateuria* sp. ANA-18, *Arthrobacter* sp. Ba-5-17, and *Acinetobacter radioresistens* (Aoki et al. 1984; Nakai et al. 1990; Kim et al. 1997; Murakami et al. 1998; Briganti et al. 2000). Comparison of the amino acid sequence of catechol 1,2-dioxygenases from reported previously, it was found that the identity of these enzymes are not high. However, structural studies of catechol 1,2-dioxygenases showed that the amino acid residues at the active site are highly conserved, especially those responsible for iron binding (Eulberg et al. 1997; Ridder et al. 1998; Vetting et al. 2000).

Several reports stated that certain soil yeasts possess a great inductive capacity for degradation of diverse aromatic compounds of small molar mass (Middelhoven 1993; Sampaio 1999), but, based on currently available literature, we found that yeasts were not only less commonly reported and in general, their tolerance and rate were inferior to those of bacteria in degrading phenol. On the other hand, although there are many reports concerning catechol 1,2-dioxygenase, they are almost (or all)

from bacteria. Reports on the purification and characterization of purified catechol 1,2-dioxygenases from eukaryotic cells are scanty or even never be cited. Therefore, how to isolate a naturally occurring yeast strain possessing a capacity to degrade effectively phenol at higher concentration for use as a prospective microorganism to degrade phenols in waste water or treatment of soil and how to purify and characterize 1,2-CTD from this eukaryotic strain are of major interest to us.

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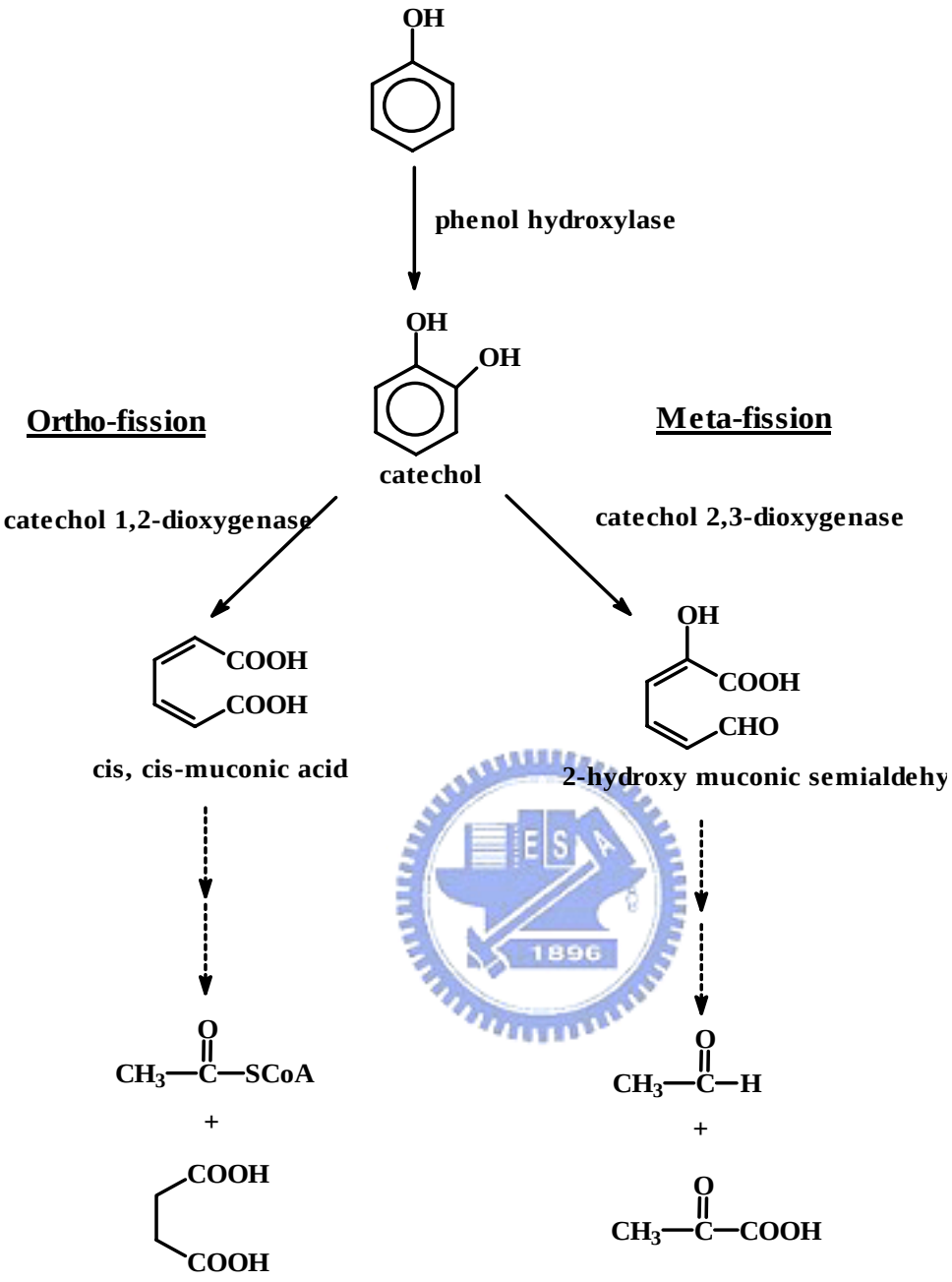


Figure 1-1. Two common phenol degradation pathways, the ortho- and meta-fission, occur in microorganisms.

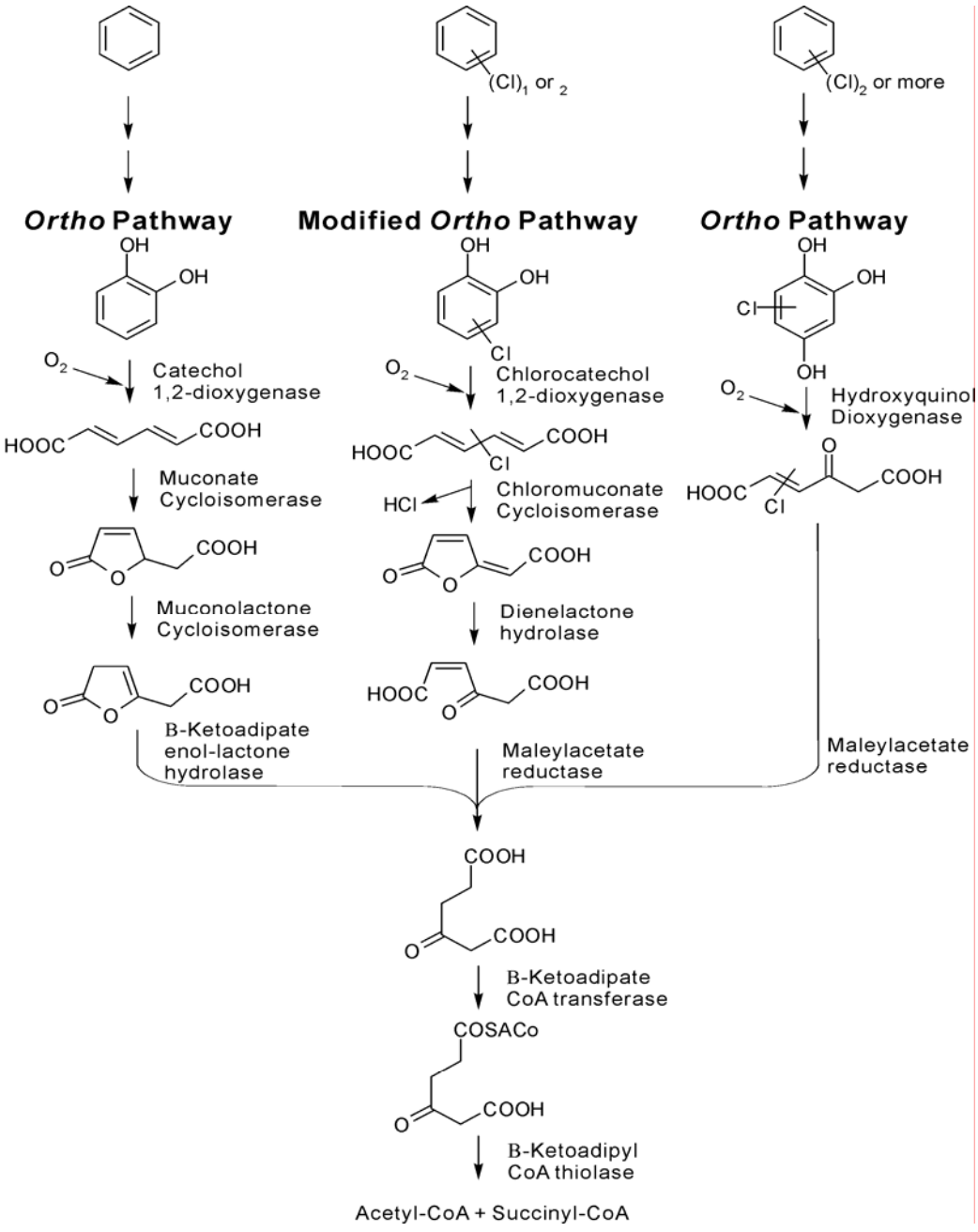


Figure 1-2. Aerobic catabolism of monoaromatic hydrocarbons

Chapter 2

Experimental

2.1 Experimental of Materials

2.1.1 Strain

The strain, *Candida albican*s TL3, used in this study was newly isolated by enrichment culture from the soil sample of a petrochemical plant in Taiwan.

2.1.2 Reagents

Formaldehyde, ammonium water and $FeSO_4$ were purchased from RDH. Catechol, protocatechuate, Sephadex G-75, TCA, urea, thiourea, TritonX-100, DTT, IPG, ammonium bicarbonate, NADPH, β-mercaptoethanol and antipyrine were purchased from Sigma. 4-Chlorocatechol and cis,cis-muconic acid were purchased from Fluka. Hydroxyquinol was purchased from Aldrich. TFA, phenol and 4-aminoantipyrene were purchased from Riedel-deHaen. Ammonium sulfate, SDS, potassium phosphate, EDTA , NaOH and glucose were purchased from J.T.Baker. Acetonitrile, coomassie brilliant R250, methanol and acetic acid were purchased from Merck. Trypsin was purchased from Promega. YNB (yeast nitrogen base), agar, tryptone and yeast extract

were purchased from Difco. FAD was purchased from CalBiochem. Agarose was purchased from USB. N,N-Methylenebisacrylamide was purchased from Amersham Pharmacia. 40 % Acrylamide and protein assay kit were purchased from Bio-Rad. Tris-(hydroxymethyl) methylamine and glycine were purchased from BDH. RGSF (RapiGest™ SF) was purchased from Waters.

2.1.3 Buffers and solution

YNB without amino acids approximate formula per liter:

Nitrogen source
- Ammonium sulfate ..... 5.0 g

Vitamins
- Biotin ..... 2.0 ug
- Calcium pantothenate ..... 400.0 ug
- Folic acid ..... 2.0 ug
- Inositol .....2,000.0 ug
- Niacin .....400.0 ug
- p-Aminobenzoic acid .....200.0 ug
- Pyridoxine hydrochloride ..... 400.0 ug
- Riboflavin .....200.0 ug
- Thiamine hydrochloride .....400.0 ug

Compounds supplying trace elements
- Boric acid .....500.0 ug
- Copper sulfate ..... 40.0 ug
- Ferric chloride ..... 200.0 ug
- Manganese sulfate .....400.0 ug
- Potassium iodide ..... 100.0 ug
- Sodium molybdate .....200.0 ug
- Zinc sulfate .....400.0 ug

Salts
- Calcium chloride ..... 0.1 g
- Magnesium sulfate ..... 0.5 g

Monopotassium phosphate ..... 1.0 g
Sodium chloride .....0.1 g

25 % YNB medium: dissolve YNB in distilled water to make a 250 mg/mL (w/v) solution, and sterilized by filtration through a 0.2 μm filter.

Culture agar: add 6g bactoagar into distilled water and make up the final volume to 400 mL, autoclave. And then dissolve 0.564 g phenol and 10.7 mL 25 % YNB medium in sufficient sterilized agar solution to bring the total volume to 400 mL.

Nitrogen base medium approximate formula per liter:

Ammonium sulfate ..... 1.0 g
Dipotassium phosphate..... 3.4 g
Monopotassium phosphate .....4.3 g
Magnesium chloride.....0.3 g
Yeast extract.....0.05 g
Calcium chloride .....13.0 ug
Ferrous sulfate .....3.0 ug
Sodium molybdate….....30.0 ug
Manganese sulfate.....5.0 ug

Phenol assay solution: mix 1% $K_3Fe(CN)_6$ (dissolve in pH 9.7, 0.1M glycine) and 1% antipyrine (dissolve in pH 9.7, 0.1M glycine) = 9 ： 1

Formaldehy assay solution: add 7.5 g ammonium acetate, 0.1 ml acerylacetone and 0.15 ml acetic acid to 100 ml distilled water.

Phenol hydroxylase assay buffer: 50 mM potassium phosphate buffer, pH 7.6, containing 170-μM phenol, 1-mM β-mercaptoethanol, 0.1-mM EDTA, 10-μM FAD and 170-μM NADPH.

Catechol 1,2-dioxygenase assay buffer: 50 mM Tris-HCl buffer, pH 8.3, containing 5-mM β-mercaptoethanol, 20-μM $FeSO_4$, and 1-mM catechol.

50 mM Buffers of pH region: pH 5.0 ~ 6.0, sodium acetate; pH 6.5 ~ 8.5, potassium phosphate; pH 9.0 ~ 10.0, Tris-acetate.

5X sample buffer (SDS-PAGE): mix 31.25 mL 1 M Tris/HCl (pH 6.8), 10 g SDS, 25 mL glycerol, 750 μL bromophenol blue (2% in ethanol), 5 μL 2-mercaptoethanol and make up the final volume to 100 mL using distilled water. Store away 5-10 ml aliquots.

Separating gel buffer: dissolve 18.2 g Tris base and 0.36 mL TEMED in 90mL distilled water and adjust the pH to 8.8 using 6N HCl. Then make up the final volume 100 mL using distilled water, stored at 4℃.

Stacking gel buffer: dissolve 0.6 g Tris base and 40 μL TEMED in 8 mL distilled water and adjust the pH to 6.8 using 6N HCl. Then make up the final volume 10 mL using distilled water, stored at 4℃.

Running buffer: 12 g Tris base, 4 g SDS, 57.6 g glycine. Adjust to pH 8.3 with 6N HCl and make up the final volume 4 L using distilled water.

0.1 % Coomassie Brilliant Blue R250: add 0.5 g Coomassie Brilliant Blue R250 200 mL methanol, then, add 50 mL acetic acid and make up the final volume to 500 mL using distilled water.

Destain solution I: mix 75 mL methanol with 100 mL acetic acid, and make up volume to 1 L using distilled water.

Destain solution II: add 200 mL acetic acid to 800 mL distilled water.

Sample buffer (2-DE): 0.5 % IPG buffer containing 6 M urea, 2 M thiourea, 0.5 % TritonX-100 and 1% DTT.

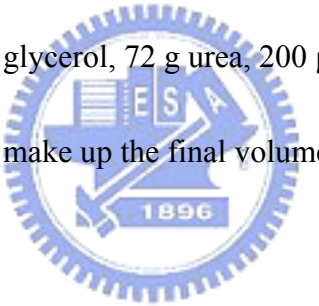
Monomer stock solution: dissolve 1.6 g N, N-Methylenebisacrylamide to 150 mL 40 % acrylamide and make up the final volume to

200 mL ddH_2O.

4X resolving gel buffer: dissolve 181.5 g Tris basen in 750 mL ddH_2O and adjust the pH to 8.8 using 6N HCl. Then make up the final volume 1 L using ddH_2O.

Agarose solution: add 0.5 g agarose to 100 mL ddH_2O. Dissolve mixture in the water bath.

SDS equilibration buffer: mix 1.5 M 10 mL Tris-HCl (pH 8.8), 4 g SDS , 69 mL glycerol, 72 g urea, 200 μL bromophenol blue and make up the final volume to 200 mL using ddH_2O.



2.1.4 Equipment

UV-Vis spectrophotometer: Shimadzu, UV-1601

Centrifuges: 1.Beckman, Allegra 21 Serias

2. Kubota

Ultrasonic processor: Sonics, VCX-750

HPLC system: Lab Alliance series

IC analyzer: DX-500 with *Gp40* gradient pump, electrochemical detector (ED40)

GC-MS: Agilent 5973 Network MSD™

FPLC system: Amersham Biosciences

Q-TOF ESI-MS (MS / MS): Micromass

ICP-MS: Agilent 7500a

IPGphor: Pharmacia

Hoefer SE 600: Amersham Biosciences

TE2 transphor electrophoresis unit: Amersham Biosciences, Hoefer

Edman sequencer: Applied Biosystems, Procise 494 sequencer

MALDI-TOF (TOF / TOF): Applied Biosystems, 4700 proteomics analyzer

2.2 Experimental of Principles

2.2.1. Experimental of Quantitative

2.2.1.1 Phenol determination (4-aminoantipyrine colorimetric method (Lacoste et al. 1959)**)**:

Phenol and 4-aminoantipyrine in an alkaline postassium ferricyanide solution form a dark red condensated product with an absorption maxima at approximately 505 nm (Fig. 2-1).

2.2.1.2 Glucose determination (GOD-PAP assay)

GOD-PAP method was developed for glucose determination. Glucose oxidase (GOD) converts the sample glucose into gluconate and hydrogen peroxide (H_2O_2) (Eq. 1). H_2O_2 was coupled to phenol and 4-aminoantipyrine, and then were catalyzed by peroxidase (POD) to a red quinone product (505 nm) (Eq. 2). The increase in OD_{505} correlates with the glucose concentration of the sample.

$$\textbf{Glucose} + \textbf{O}_2 \xrightarrow{GOD} \textbf{Gluconate} + \textbf{H}_2\textbf{O}_2 \text{ - - - - - - - -(Eq. 1)}$$

$$2\,\textbf{H}_2\textbf{O}_2 + \textbf{Phenol} + \textbf{4-Aminoantipyrine} \xrightarrow{POD} \textbf{Red quinone} + 4\,\textbf{H}_2\textbf{O} \text{ - -(Eq. 2)}$$

2.2.1.3 Formaldehyde determination (Hantzsch reaction) (Nash 1953):

The mean of Hantzsch reaction usually used for colorimetric estimating formaldehyde concentration**.** When traces of formaldehyde are added to

approximately neutral solutions of acetylacetone and ammonium salt, a yellow color gradually develops owing to the synthesis of diacetyldihydrolutidine (DDL).

2.2.1.4 Protein determination:

Scopes method (Scopes 1974) — Using the below equation (Eq. 3), to obtain a value for the extinction coefficient of a protein at 205 nm which gives results mostly correct to within 2 % when used for estimating protein concentration**.**

$$\epsilon_{205}^{1\ mg/ml} = \frac{27.0}{1 - 3.85 \times (A_{280}/A_{205})} \quad \text{---------(Eq. 3)}$$

Bradford method (Bradford 1976) — The protein concentration was estimated by the dye binding method of bicinchoninic acid (BCA) and using bovine serum albumin as a standard. This method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (562 nm) using an unique reagent that contains bicinchoninic acid.

2.2.2 Experimental of Chromatographic separations

2.2.2.1 Ion chromatography (IC)

Ion chromatography is a special form of liquid chromatography where charged species are separated by selective distributions in an electrolytic mobile phase and a stationary phase with weak ionic sites. Detection in Ion Chromatography is usually

performed by a conductivity detector - the greater the concentration of ions the higher the conductivity of the solution. However, because the mobile phase consists of an electrolyte, the conductivity of the mobile phase itself is quite high. This results in a large background current that must be dealt with in order to make sensitive measurements. The "suppressor" system neutralizes the mobile phase salts thereby lowering the background current.

2.2.2.2 Reverse phase high-performance liquid chromatography (RP HPLC)

Reverse Phase HPLC is one of common form of chromatography used in compounds separation. Compounds stick to reverse phase HPLC columns (stationary phase is generally made up of hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$)) in high aqueous mobile phase and are eluted from RP HPLC columns with high organic mobile phase. In RP HPLC compounds are separated based on their hydrophobic character. Sample can be separated by running a linear gradient or isocratic mode of the organic solvent.

2.2.2.3 Q sepharose chromatography

Q sepharose (strong anion exchanger) chromatography separates proteins with differences in charge. The separation is based on the reversible interaction between a charged protein and an anion exchanger (stationary phase). Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are

eluted differentially. This elution is usually performed by increases in salt concentration or changes in pH. Most commonly, samples are eluted with salt, using a gradient elution.

2.2.2.4 Gel filtration chromatography

Gel filtration chromatography separates proteins with differences in size. In this method, fine, porous beads are packed into a chromatography column. When proteins are loaded onto the column, larger molecules are excluded from the gel beads and emerge from the column sooner than smaller molecules, whose migration is retarded because they can enter the beads.

2.2.3 Experimental of Mass Spectrometry Methods

2.2.3.1 Gas chromatography-mass spectrometry (GC-MS)

GC-MS is a candidated technique use for the analysis of volatile compounds such as catechol. Capillary GC is a separation technique in the gaseous phase where a sample (which is most often derivatized) passes through capillary tubes whose inner walls contain a thin film of an adsorbing medium. When the components are eluted they may be detected by mass spectrometery. The MS performs either electron impact (EI) or chemical ionisation (CI) on the elutant in order to produce a mass spectrum.

This spectrum is then used to identify the components of the sample by compound library searching (e.g., via NIST which contains 80,000 spectra).

2.2.3.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

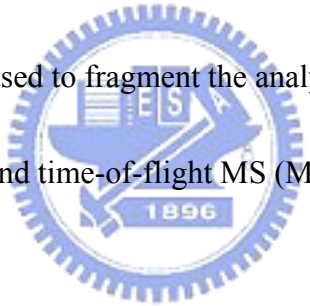
ICP-MS is a very powerful tool for trace (ppb-ppm) and ultra-trace (ppq-ppb) elemental analysis. In ICP-MS, a plasma or gas consisting of ions, electrons and neutral particles is formed from Argon gas. The plasma is used to atomize and ionize the elements in a sample. The resulting ions are then passed through a series of apertures (cones) into the high vacuum mass analyzer. The isotopes of the elements are identified by their mass-to-charge ratio (*m/e*) and the intensity of a specific peak in the mass spectrum is proportional to the amount of that isotope (element) in the original sample.

2.2.3.3 Quadrupole-time of flight electrospray ionization-mass spectrometry and **tandem mass spectrometry (Q-TOF ESI-MS** and **MS/MS)**

ESI-MS is one of the two most common methods of mass spectrometry for protein analysis. In this MS method the ionization process is carried out at atmospheric pressure (API), and involves spraying a solution of the sample in a suitable solvent out of a small needle, to which a high voltage is applied (Fig. 2-2) (http://qbab.aber.ac.uk/roy/mss/qtof.htm). This process produces small charged droplets, and the solvent is the evaporated leaving the sample molecule in the gas phase

and ionized. This is then 'swept' into a MS that is held essentially *in vacuo* and the ions separated and detected using a reflectron-based time-of-flight (TOF) analyser. The attachment of many protons per protein molecule leads to a series of m/z peaks for this single protein. By computer analysis of the data from this series of peaks that generates a single peak at the correct molecular mass of the protein.

In this MS-MS approach (depicted below) a first mass spectrometer (MS-1) that employs a quadrupole mass filter is tuned to allow only the analyte ion of interest through (Fig. 2-3) (http://qbab.aber.ac.uk/roy/mss/qtof.htm). This is then taken into a collision cell where Argon is used to fragment the analyte, and the so-called daughter ions are then swept into a second time-of-flight MS (MS-2) where they are separated and detected.



2.2.3.4 MALDI-TOF-MS and MALDI-TOF/TOF-MS

MALDI-TOF-MS is another of the two most common method of mass spectrometry for protein analysis. In this MS method a solid matrix is used, which absorbs light at the wavelength the laser produces (Fig. 2-4) (http://qbab.aber.ac.uk/roy/mss/qtof.htm). The sample (e.g., the fragments of protein spot from a 2-D gel are excised and digested with a specific protease) is mixed with a matrix solution and allowed to co-crystallise on a target plate. When the laser is fired

at the target the matrix absorbs the laser light energy which vaporizes it (it desorbs from the surface) and this carries some of the sample with it. At the time that the laser is pulsed a voltage is applied to the target plate to accelerate the ionised sample towards a time-of-flight (TOF) mass analyser. This peptide mass fingerprint can then be used to search databases to identify the protein. The precision is considered to be approx 10 ppm.

In this tandem MS approach that there are an ion selecting filter (allow only the peptide fragment ion of interest through) and a collision cell between TOF-1 and TOF-2. The *de novo* sequencing of this interested peptide can be predicted by analysis of the special computer software.

2.2.4 Others

2.2.4.1 Salting-out

The solubility of protein depends on the salt concentration in the solution. As the salt concentration is reached to the point of maximum protein solubility. Further increase in the salt concentration, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as *salting-out*. Among of

these salts, ammonium sulfate has been the most widely used to effect protein separation and purification through salting-out.

2.2.4.2 Edman sequencing

Edman sequencing is known as N-terminal sequencing of a protein. In this technique, the N-terminal amino acid is derivatized with PITC (phenylisothiocyanate), cleaved by acid and identified by reverse phase chromatography. Repeating this process gives a protein sequence.

2.2.4.3 Two-dimensional gel electrophoresis

Two-dimensional (2D) separation involves first separating protein based on their isoelectric point (pI) using isoelectric focusing (IEF). The isoelectric point is the pH at which there is no net electric charge on protein. IEF is an electrophoretic technique whereby proteins are separated in a pH gradient. An electric field is applied to the gradient and protein migrate to the position in the pH gradient equivalent to the pI (Gorg et al. 2000).

The second step in 2D gel electrophoresis is to separate proteins based on molecular weight using SDS-PAGE. Individual proteins are then visualized by Coomassie or silver staining techniques or by autoradiography. Because 2D gel electrophoresis separate proteins based on independent physical characteristics, it is a

powerful means to resolve complex mixtures proteins. Modem large-gel formats are reproducible and are the most common method for protein separation in proteomic studies.

2.3 Experimental of Methods

2.3.1 Phenol determination

Mix 50 μL supernatant sample with 950 μL phenol assay solution at 30℃ and measure spectrophotometrically absorbance at 505 nm after a 30 min incubation.

2.3.2 Formaldehye determination

Mix 10 μL supernatant sample with 990 μL formaldehyde assay solution at 50 ℃ and measure spectrophotometrically absorbance at 412 nm after a 5 min incubation.

2.3.3 Glucose determination

Mix 50 μL supernatant sample with 950 μL GOD-PAP assay buffer at 37 ℃ and measure spectrophotometrically absorbance at 505 nm after a 5 min incubation.

2.3.4 Protein determination (Bradford method)

Mix 10 μL supernatant sample with 990 μL protein assay solution at 37 ℃ and measure spectrophotometrically absorbance at 562 nm after a 30 min incubation.

2.3.5 Phenol hydroxylase activity assay

Mix 30 μL supernatant crude enzyme extract with 970 μL phenol hydroxylase assay buffer at 25 ℃ and measure spectrophotometrically absorbance at 340 nm per

min (Hayaishi et al. 1957).

2.3.6 Catechol 1,2-dioxygenase activity assay

Mix 2 (or 10) μL supernatant crude enzyme extract with 998 (or 980) μL catechol 1,2-dioxygenase assay buffer at 25 ℃ and measure spectrophotometrically absorbance at 260 nm per min (Varga and Neujahr 1970).

2.3.7 In-solution digestion

1. Dissolve 300 μg of target protein in 700 μL of ABC solution and 200 μL of RGSF solution, in a 1.5-mL plastic microcentrifuge tube.
2. Add 100 μL of DTT solution and mix the sample by gentle vortex.
3. Reduce the protein mixture for 30 min at 60℃.
4. Add 200 μL of IAA solution and mix the sample by gentle vortex.
5. Alkylated the protein mixture for 30 min at room temperature and avoid light.
6. Add 100 μL of trypsin solution and mix the sample by gentle vortex. Then, carry out the digestion for 37℃, 18 h.
7. Adding 120 μL of HCl to stop the reaction (37℃, 45 min).
8. Centrifugation and filtration of the solution for HPLC separation. Table 2-1 list the in-solution digestion compositions.

2.3.8 In-gel digestion

1. Transfer the gel slices (protein sports) from 2-D gel into a cleaned 650 microcentrifuge PP tube (siliconized, methanol washed). It is better to cut the protein sports into 1 mm^3 pieces.

2. Add 100 μL of 50% acetonitrile / 25 mM ammonium bicarbonate buffer (pH 8.5). Soak for 15 min. Centrifuge at 13000 rpm for 1 min. Remove buffer completely. Repeat this step twice or more times.

3. Soak the gel in 100 μL of 100 % acetonitrile for 5 min (allow gel slices turn white) and centrifuge at 13000 rpm for 1 min. Remove acetonitrile.

4. Dry the gel slices for about5 min in a speed vac.

5. Rehydrate the gel slices with 0.1 μg trypsin in 10 μL 25 mM ammonium bicarbonate buffer (pH 8.5).

6. Crash the gel slices with a cleaned siliconized PP microcentrifuge pestle.

7. Spin down the gel pieces / trypsin solution and incubate at 37 °C for 16 h.

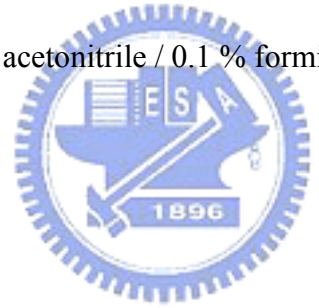
8. Add 50 μL of 50 % acetonitrile / 5 % TFA to sample gel. Sonicate sample gel for 10 seconds, and then stop for 10 seconds. Repeat sonication 10 times.

9. Spin down the gel pieces at 13000 rpm for 1 min. Aspirate the supernatant containing peptide mixtures from the sample tube and transfer to the corresponding new tube.

10. Repeat step 8 to 9. Combine the two extract peptide solutions and concentrate in a speed vac. at 35 °C to 1-2 μL.

11. Redissolvation:

It is recommended to dissolve the sample with 1 μL of 1 % formic acid first, then add 9 μL 50 % acetonitrile / 0.1 % formic acid and sonicate for 30 seconds.



2.3.9 SDS-PAGE

SDS-PAGE is performed essentially according to the method of Dais and Laemmli using a gel system (mightly Small II SE250). In SDS-PAGE, the protein sample is diluted in sample buffer (5X) at room temperature and a constant voltage of 150 V. Table 2-2 list the SDS-PAGE compositions.

2.3.10 2-D PAGE

First dimension (isoelectric focusing)

1. Pipettle sample buffer mixed with sample into the strip holder. Then, place t

he IPG strip gel into the strip holder with the dried gel side down avoiding air bubbles. Pipettle paraffin oil over the strip to avoid strip dry out or urea crystallize out.

2. Rehydration of the IPG strip loading under 30 V and focusing for 12 h in the strip holder on the IPGphor system. Then, instrument setting as the following program for IEF separation.

Voltage	Time
30 V	12 h
500 V	2 h
1000 V	2 h
8000 V	4 h

Second dimension (SDS polyacrylamide gel electrophoresis)

1. When IEF run is finished, the IPG strip is directly transferred to SDS equilibration buffer with 1% DTT or 2.5 % iodoacetamine for 12-15 min, and further undergo second dimension electrophoresis.

2. The IPG strip is placed on the SDS gel and overlayed with agarose solution.

3. SDS-PAGE run about 5 h 30 min under 200 V.

2.3.11 Coomassie blue staining

After electrophoresis, the gel is soaked in Coomassie blue R250 staining

solution for 20 min. Then the gel is destained with destaining solution I for 20 min twice times and continue to destain the gel with destaining solution II until the stained band (or sport) is distinct against a clear background.

2.4 References

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Table 2-1. Compositions of in-solution digestion.

Reagents	Concentration
Ammonium bicarbonate	25 mM
RGSF	5 X
DTT	50 mM
IAA	100 mM
Trypsin	100 ng / μL
HCl	500 mM

Table 2-2. Compositions of SDS-PAGE.

Solution	Separating gel	Stacking gel
	12.5%	4%
Monomer stock solution	3.1 mL	0.5 mL
Separating gel buffer	2.5 mL	—
Stacking gel buffer	—	1.24 mL
10% SDS	0.1 mL	0.05 mL
Distilled H_2O	4.25 mL	3.11 mL
APS	0.05 mL	0.1 mL
Total volume	10 mL	5.0 mL

Table 2-3. Compositions of 2-D PAGE.

Solution	12.5%
Monomer stock solution	20.8 mL
4X resolving gel buffer	12.5 mL
TEMED	16.5 μL
10% SDS	0.5 mL
ddH_2O	15.9 mL
10% APS	250 μL
Total volume	50 mL

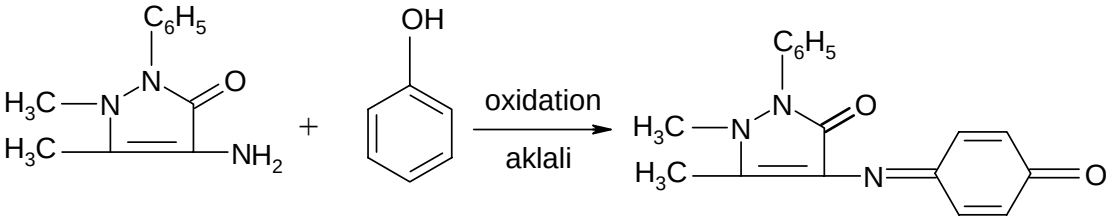

Figure 2-1. Condensational reaction of phenol and 4-aminoantipyrine.

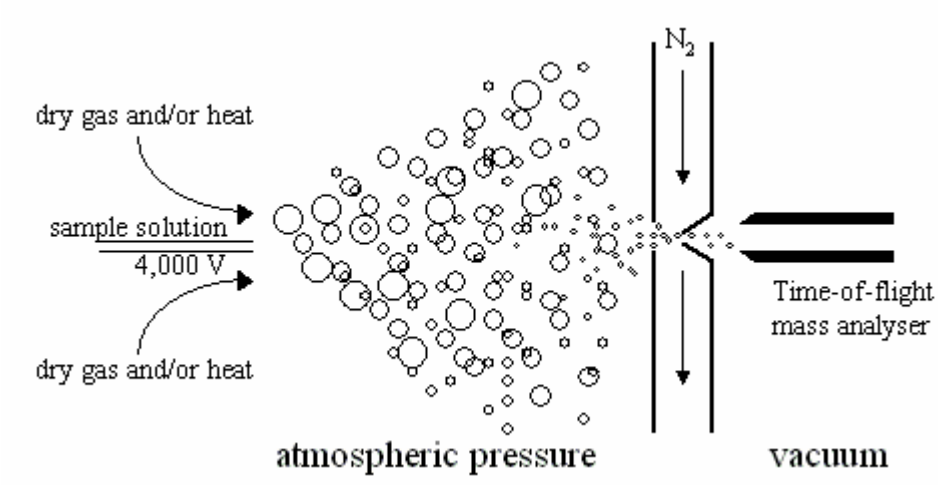

Figure 2-2. A process of ESI.

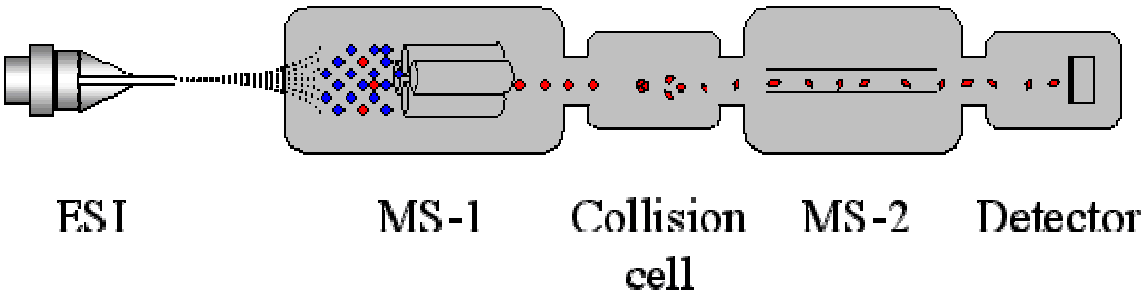

Figure 2-3. Configuration used in Q-TOF ESI-MS/MS.

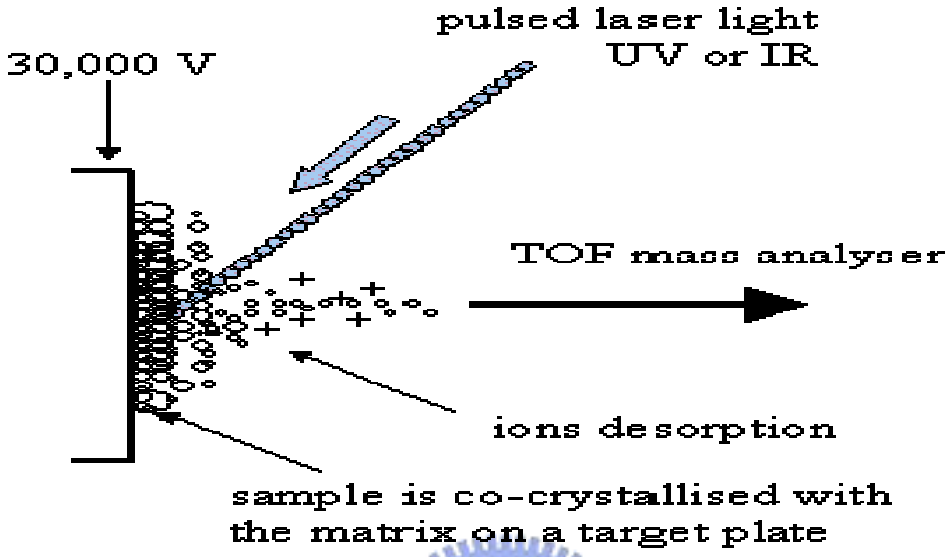

Figure 2-4. A process of MALDI-TOF.

Chapter 3

An isolated *Candida albicans* TL3 capable of degrading phenol at large concentration

3.1 Abstract

An isolated yeast strain was grown aerobically on phenol as a sole carbon source up to 24 mM; the rate of degradation of phenol at 30 ^{o}C was greater than other microorganisms at the comparable phenol concentrations. This microorganism was further identified and is designated *Candida albicans* TL3. The catabolic activity of *C. albicans* TL3 for degradation of phenol was evaluated with the K_S and V_{max} values of 1.7 ± 0.1 mM and 0.66 ± 0.02 μmol/min/mg of protein, respectively. With application of enzymatic, chromatographic and mass-spectrometric analyses, we confirmed that catechol and *cis, cis*-muconic acid were produced during the biodegradation of phenol performed by *C. albicans* TL3, indicating the occurrence of an ortho-fission pathway. The maximum activity of phenol hydroxylase and catechol-1,2-dioxygenase were induced when this strain grew in phenol culture media at 22 mM and 10 mM, respectively. In addition to phenol, *C. albicans* TL3 was effective in degrading formaldehyde, which is another major pollutant in waste water from a factory producing phenolic resin. The promising result from the bio-treatment of such factory effluent makes *Candida albicans* TL3 be a potentially

useful strain for industrial application.

Keywords: phenol degradation, phenol hydroxylase, catechol-1,2-dioxygenase, catechol, *cis, cis*-muconic acid

3.2 Introduction

Phenol and formaldehyde, hazardous pollutants in the environment throughout the last century, are present in effluents from many industries. Physical and chemical methods to treat organic compounds require many processing steps, which are costly and typically produce also other toxic end products (Kobayashi and Rittmann 1982). Biodegradation has been considered to be a highly effective method of decontamination of a fouled environment. The remarkable ability of microbes to decompose chemicals not only is useful in pollution remediation but also serves as a prospective tool to detect pollutants.

Many microorganisms, including bacteria (Hughes and Bayly 1983; Gurujeyalakshmi and Oriel 1989; Rahalkar et al 1993; Yap et al 1999; Glancer-Soljan et al 2001; El-Sayed et al 2003; Chen et al 2004; Margesin et al 2005), yeasts (Kato et al 1982; Bastos et al 2000; Fialova et al 2004), algae (Semple and Cain 1996) and aquatic fungi (Ristanovic 1975), are found to be capable of degrading phenol or formaldehyde at various concentrations. Since 1985, the catabolism of aromatic hydrocarbons by microbes has been widely discussed. The typical pathway of phenol degradation in microorganisms occurs via the formation of catechol derivative following by ring cleavage through the ortho-fission or meta-fission pathway (Yang

and Humphrey 1975). Both pathways commonly use phenol hydroxylase, a monoxygenase, as catalyst at first step of degradation. However, they are different in the second degradation step (Fig. 1-1). In general, yeasts and most bacteria utilize catechol 1,2-dioxygenase and 2,3-dioxygenase, corresponding to the ortho-fission and the meta-fission pathway, respectively, to perform the oxidative reaction of catechol (Neujahr and Gaal 1973; Muller and Babel 1994). There were some literatures reported that certain soil yeasts possess a great inductive capacity for degradation of diverse aromatic compounds with low molecular weight (Middelhoven 1993; Sampaio 1999), but, based on the previous reports, we found that yeasts were less commonly reported than bacteria. We are interested in how to screen a natural occurring yeast possessing a capacity to tolerate and effectively degrade phenol at higher concentration for use as a prospective microorganism in waste water or soil pollution remediation. In this work, we characterized the capacity of an isolated strain, *Candida albicans*, to degrade not only phenol but also formaldehyde, another toxic pollutant. A strain with effective operation on both toxic ingredients has been rarely reported. We discuss here the phenol catabolic pathway of *C. albican* that we investigated, and also evaluated the efficiency of *C. albicans* TL3 to treat waste water from a factory producing phenolic resin.

3.3 Experimental

3.3.1 Media formulation and microorganism screening

The microorganism used in our work was isolated from the soil sample of a petrochemical plant in Taiwan. Soil sample was stirred for 2 h at room temperature in sterile distilled water. 5 ml of the above sample was inoculated in 150 ml of YNB medium (0.67 % w/v) containing various phenol concentrations (10, 12, 14, 16, and 18 mM). After 2 weeks of cultivation (150 rpm, 30℃), 0.1 ml of the resulting culture medium (OD_{600} of 1.65) was subjected to spreading on agar plates containing the sameYNB medium with 15 mM phenol. Strains were obtained after purification by sequential cultures. A powerful phenol-degradation strain was identified as *Candida albicans* by CBS (Centralbureau voor Schimmelcultures, Boarn, Netherlands) based on its morphology, physiological and biochemical characteristics. To investigate a carbon source, we directly added a suitable amount of phenol, glucose, or *cis, cis*-muconic acid to YNB culture medium (0.67 %). All resulting media were sterilized by ultra-filtration before use. Unless otherwise stated, all flask cultures were incubated in a rotary shaker (150 rpm) at 30℃.

3.3.2 Cell growth and Phenol degradation

The isolated strain was cultured at 30℃ in flask containing YNB medium (50 mL,

0.67 %) with phenol at various concentrations (0 – 24 mM) and/or glucose (0.2%, w/v). To examine the prospective application of this microbe, we used waste water obtained from a local manufacturer of phenolic resin (Chang Chun Plastics Co., Ltd., Taiwan) as a carbon source and added it directly to YNB culture medium (0.67 %). We withdrew samples from the cultures at various intervals, and estimated the cell density by measurement of the absorbance of the sample at 600 nm using a UV-Vis spectrophotometer (Shimadzu, UV-1601) and with a colorimetric method, using 4-aminoantipyrene to estimate the residual phenol in the culture medium (Lacoste 1959). The residual glucose and formaldehyde were determined by GOD-PAP assay and Hantzsch reaction (Nash 1953), respectively. All cultures were cultivated with an initial cell density 0.02 (OD_{600}) per milliliter of medium in triplicate; the mean values are reported, for which values of standard error were less than 10 %.

For measuring the kinetic parameters of the whole-cell, different phenol concentrations (0.5-5 mM) were prepared in 1 ml culture medium (50 mM Tris, pH 7.0) containing the final cell density (stationary phase) of OD_{600} of 1. The resulting mixture was incubated at 30℃ with an agitation rate of 150 rpm. The residual phenol concentration was monitored after the inoculation of microorganism, with which the initial rate of phenol degradation can be obtained. Data were analyzed by non-linear regression (Grafit program) using the Haldane's equation (Folsom 1990)

shown as below.

$$v = [\ V_{max} \cdot (S)] / [(S) + K_S + (S)^2 / K_I]$$

Where v is the initial rate of degradation, (S) is the phenol concentration, K_S is the half-saturation constant (equivalent to K_m in enzyme kinetics), K_I is the inhibition constant, and V_{max} is the theoretical maximum degradation rate.

3.3.3 Enzyme activity assays

A cell culture (50 mL) was harvested from YNB medium (0.67 %) containing phenol at various concentrations when the growth of *C. albicans* TL3 approached a stationary phase. After centrifugation, a cell pellet were washed twice with distilled water (2 mL) and then resuspended in the assay buffer (0.5 mL, 50-mM potassium phosphate buffer, pH 7.6, containing 1-mM β-mercaptoethanol, 0.1-mM EDTA and 10-μM FAD). After disruption by sonication, the cell debris was removed by centrifugation at 13700 *g* for 30 min at 4℃. The supernatant (crude enzyme extract) was used for assay of enzymatic activity, metabolite preparation and determination of protein concentration. Phenol hydroxylase (EC 1.14.13.7) activity was assayed spectrophotometrically on monitoring the disappearance of NADPH in absorbance at 340 nm (Hayaishi 1957). Catechol 1,2-dioxygenase (EC 1.14.13.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) activities were assayed by determining the rate of

accumulation of *cis*, *cis*-muconic acid (absorbance increase at 260 nm) (Varga and Neujahr 1970) and 2-hydroxymuconic semialdehyde (absorbance increase at 375 nm) (Sala-Trepat and Evans 1971), respectively. Control reactions (without substrate or crude enzyme extract) were performed for each assay. One unit of phenol hydroxylase is defined as the amount of enzyme that catalyzes the disappearance of NADPH at 1 μmol min^{-1}. One unit of catechol 1,2-dioxygenase is defined as the amount of enzyme that catalyzes the formation of *cis, cis*-muconic acid at 1 μmol min^{-1}. The concentration of protein was determined with a protein assay kit (Bio-Rad) with bovine serum albumin as standard; the specific activity is defined in units per mg protein.

3.3.4 Product analysis and identification

To prepare the metabolites, we added crude enzyme extract (described in the previous section, 30 μL) to reaction mixture (970 μL, 50-mM potassium phosphate buffer, pH 7.6, containing 170-μM phenol, 1-mM β-mercaptoethanol, 0.1-mM EDTA, 10-μM FAD and 170-μM NADPH). The resulting mixture was incubated for 10 or 25 min at 25℃. The reaction was terminated on addition of methanol (1 mL). After removing the protein precipitant by centrifugation, we analyzed the supernatant by HPLC. The suspected product fractions were collected and used for GC-mass

analysis. For analysis by HPLC (Lab Alliance series 4 pump, detector: GL Sciences UV-620) we used a reversed phase column (Waters, *u* Bondapak $C_{18,}$ 3.9×300mm; mobile phase, methanol: 1% acetic acid = 80:20 v/v), at a flow rate 1 mL min^{-1}, and monitored the compounds of the supernatant at 260 nm. To prepare the enzymatic products of catechol-1,2-dioxygenase, we added crude enzyme extract (10 μL) to reaction mixture (990 μL, 50-mM Tris-HCl buffer, pH 8.3, containing 5-mM β -mercaptoethanol, 20-μM $FeSO_4$, and 1-mM catechol) and incubated for 2 or 10 min at 25℃. The reaction was terminated on addition of methanol (1 mL). The resulting protein precipitant was removed by centrifugation. We analyzed the supernatant by ion chromatography (IC), and collected the product fractions for use in LC mass and LC tandem mass-spectrometric analysis. We performed ion-chromatographic analysis (DX-500 with *Gp40* gradient pump) on a column (DIONEX, Ion Pac Asll, 4x250mm; mobile phase, NaOH solution 0.01 M, flow rate 1.5 mL min^{-1}) and monitored the conductivity of compound with an electrochemical detector (ED40) and an anion self-regenerating suppressor set at 50 mA.

3.3.5 Mass-spectrometric analysis

For GC-MS (ion trap detector, Perkin-Elmer Instruments, Turbo Mass Gold Mass spectrometer, with a MDN-5ms 30-mm column, inner diameter 0.25 mm, film

thickness 0.25 μm, MDN, Supelco, PA, USA), the analytical conditions were70℃ for 2 min, 70-250℃ at 20℃ per min and 250℃ for 5 min. The temperatures of the injector, ion source and transfer lines were 250℃, 200℃ and 220℃, respectively. The mass spectra were recorded on a time-of-flight mass spectrometer (Q-TOF Micromass) in the electrospray (-) mode. The mass analyzer was scanned over a ratio m/z of mass to charge 50-500 u, with a scan step 2 s and an inter scan 0.1 s/step. The source block temperature and desolvation temperatures were set at 80℃ and 150 ℃, respectively. The rate of flow of the delivery solvent (10 % acetonitrile containing 0.1 % aqueous NH_3) was 1mL min^{-1}.

3.4 Results and discussion

3.4.1 Identification of the isolated strain and its tolerance against phenol

Because of the industrial demand of phenol degradation, many microbes have been screened. Potentially useful strains might be subjected to random mutagenesis to improve the degrading power (Chang et al 1995; Yap et al 1999). Table 3-1 summarizes the capability of various yeasts to degrade phenol completely (Neujahr and Varga 1970; Hirayama et al. 1994; Chang et al 1995; Bastos et al. 2000; Santos and Linardi et al. 2001; ,Fialova et al. 2004; Margesin et al. 2005). Most native yeasts can tolerate phenol at a concentration no greater than 18 mM. With much effort in mutagenesis, *Candida tropicalis* was improved to accept a phenol concentration up to 22 mM (Chang et al 1995). Using phenol enrichment, we screened a few microbial strains with varied ability to utilize phenol as a carbon source. Among them, *Candida albicans* was identified (further designated TL3) (Table 3-2) and evaluated as having the greatest potential to degrade phenol. Without further mutagenic treatment, *Candida albicans* TL3 tolerates phenol up to 24 mM (~2400 ppm). This record is not only the greatest among yeasts but also greater than most bacteria isolated in the environment, according to current literature (Jeong et al. 1998; Yap et al 1999; Bastos et al. 2000; Margesin et al. 2005). The ability of

C. albicans to degrade phenol that we discovered is first reported here.

In addition to phenol, potential metabolites such as catechol and *cis, cis*-muconic acid were tested as a source of carbon for *C. albicans* TL3 (Table 3-2). The results show that catechol was effectively digested, but *cis*-muconic acid and other compounds remained intact.

3.4.2 Cell growth and phenol degradation

To understand further the catabolic property of *C. albicans* TL3**,** we investigated the microbial growth with various carbon sources. Fig. 3-1, 2 shows the temporal course of replicated biomass population and the simultaneous phenol degradation by *C. albicans* TL3 in a liquid medium with phenol and/or glucose at various concentrations. As shown in Figure 3-1, increasing the concentration (5 – 24 mM) of phenol in the culture medium evidently produced a prolonged lag for *C. albicans* TL3 to adapt to the growth environment and consequently resulted in a delay to attain the stationary phase of cell growth. The cell growth approached the stationary phase within four days with the amended substrates, except for the case of phenol (24 mM), for which incubation for ten days was required. The temporal course of substrate degradation (Figure 3-2) correlated well with cell growth. At the stationary phase, the biodegradation of phenol was nearly complete (>99 %). When

glucose (0.2 %) was used as the sole nutrient, *C. albicans* TL3 grew rapidly with a log-phase feature, whereas in a mixed substrate system (15-mM phenol + 0.2 % glucose) the cell grew with a two-stage feature, shown as Figure 3-1. Glucose was consumed rapidly in the first stage of cell growth, whereas, phenol, serving as nutrient in the second growth stage, was nearly unchanged in the first stage (Fig. 3-2). Although *C. albicans* TL3 can use phenol as a source of energy, the presence of phenol could inhibit cell growth in a glucose medium [*c.f.* the growth curves of 0.2% glucose with and without phenol in Fig. 3-1]. Based on the rate of substrate disappearance, we conclude that *C. albicans* TL3 favors utilization of glucose over phenol when both ingredients exist together. The effects of glucose on phenol degradation were, however, dependent on the inoculated microbial strains. For instance, the preference for glucose metabolism of *C. albicans* TL3 was similar to that of *Trichosporon cutaneum* sp. LE3 (Santos and Linardi 2001), but different from that of another strain of *T. cutaneum* (Skoda and Udaka 1980) and *C. maltosa* (Hofmann and Vogt 1987). As discussed in the literature (Gaal and Neujahr 1981), the presence of glucose and thereafter its metabolites might inhibit *C. albicans* TL3 from the transport of phenol or the synthesis of such a transport system. Most mesophilic yeasts that are highly active in degrading phenol, such as *Candida tropicalis* (Bastos et al. 2000), *Candida maltosa* (Fialova et al. 2004) and *Trichosporon* spp. (Santos and

Linardi 2001) are reported to degrade phenol (16-18 mM) already within 5-6 days at 30℃, but *C. albicans* TL3 needed only three days to consume phenol (20 mM) at the same temperature (Figure 3-2). Some mesophilic bacteria with great activity to degrade phenol, such as *Burkholderia cepacia* PW3 and *Pseudomonas aeruginosa* AT2 (El-Sayed et al. 2003) were reported to degrade phenol (22 mM) within 11-15 days at 30℃, but *C. albicans* TL3 required only four days at the same temperature and concentration of phenol (Fig. 3-2). These results show that the rate of degradation of phenol by *C. albicans* TL3 is clearly greater than that of other microorganisms effective in degrading phenol.

The kinetic property of phenol degradation catalyzed by *C. albicans* TL3 gave a K_S and a V_{max} value of 1.7 ± 0.1 mM and 0.66 ± 0.02 μmol/min/mg of protein, respectively (Fig. 3-3). The K_S and V_{max} values are higher than those for several *Pseudomonas* strains (Folsom et al. 1990; El-Sayed et al. 2003) and *Burkholderia cepacia* (El-Sayed et al. 2003). Phenol also exhibited a weak inhibition with a K_I value of 40 ± 8 mM, which is much higher than those of *Pseudomonas* strains (Folsom et al. 1990; El-Sayed et al. 2003) and *Burkholderia cepacia* ranging from 1 – 17.8 mM (El-Sayed et al. 2003).

3.4.3 Effect of temperature and nitrogen bases on the growth of *C.albicans* TL3

The growth of *C. albicans* TL3 in phenol (15 mM) at 25, 30, 35 and 40℃ was monitored (Figure 3-4). After incubation (48 h), the cell cultures at 30 and 35℃ approached a stationary phase, whereas the cell density of cultures at 25 and 40℃ were only approximately 33 % and 7 % of that at 30℃ (or 35℃), respectively. The cell growth temperature was therefore controlled at 30℃ for further enzymatic tests. Another, considering the effect of various nitrogen bases on the growth rate of *C. albicans* TL3, while ammonium sulfate nitrogen base instead of YNB, similar growth rate were observed (Fig. 3-5).

3.4.5 Characterization of the pathway of phenol degradation by *C. albicans* TL3.

To assay the activity of phenol-degrading enzyme, we prepared the crude enzyme extract by ultra-sonication of the *C. albicans* TL3 cell cultivated to the stationary phase with phenol at various concentrations. The specific activities of phenol hydroxylase and catechol 1,2-dioxygenase of the crude enzyme extract were examined and are summarized in Table 3-4. In general, significant activities of phenol hydroxylase and catechol 1,2-dioxygenase are detectable except the case of using glucose (0.2 %) alone as nutrient. The activity of catechol 2,3-dioxygenase was not detected throughout these tests. Although the strain required phenol as an inducer to initiate the biosyntheses of phenol hydroxylase and

catechol-1,2-dioxygenase, phenol at a greater concentration suppressed the biosynthesis of both enzymes. For instance, phenol hydroxylase and catechol 1,2-dioxygenase activity began to decline when phenol was employed at concentrations >22 mM and >10 mM, respectively. When the strain was grown in a medium containing phenol (15 mM) and glucose (0.1%, 0.2% and 0.4%), the specific activities of phenol hydroxylase were approximately 70, 34 and 17 % of that of the culture in medium with phenol as sole carbon source, respectively. Similarly, the specific activities of catechol 1,2-dioxygenase were 1.25, 1.46 and 1.70-fold smaller in a phenol medium containing glucose at 0.1%, 0.2% and 0.4%, compared with that without glucose. The biosynthetic paths of phenol hydroxylase and catechol 1,2-dioxygenase in this microbe are hence likely modulated by one or more additional regulatory mechanisms such as catabolite repression.

The effect of temperature on enzyme induction was also investigated. The activities of phenol hydroxylase and catechol 1,2-dioxygenase of *C. albicans* TL3 cultivated at temperatures 25, 30, 35 and 40℃ are summarized in Table 3-5. A trend is observable that a lower temperature seemed to be favorable to induce both enzyme activities. The optimal growth temperature for *C. albicans* TL3 in phenol was near 30-35℃, but both enzyme activities decreased inversely with growth temperature. As the temperature factor on the rate of cell growth and the induction

of phenol hydroxylase and catechol 1,2-dioxygenase were discordant, both enzymes were unlikely to play a major role in rate of growth of *C. albicans* TL3.

To identify the phenol metabolic pathway in *C. albicans* TL3, we isolated the metabolites of phenol and analyzed them with a mass spectrometer. Fig. 3-6 shows overlaid HPLC chromatograms with samples having various compositions (such as NADPH and phenol) and enzymatic product(s). The chromatograms labeled a, b and c in figure 3-6 exhibited the analytical outcomes of samples without both NADPH and phenol, with NADPH, and with phenol, respectively. Discernible in fig. 3-6 (d-f), an additional feature with a retention period 3.1 min evolved when both NADPH and phenol were added in the assay solution. This feature is suggested to be catechol, according to spiking catechol (0.02 mM) in the sample (chromatogram e in Fig. 3-6). The area of the feature for a sample incubated 25 min (chromatogram f) is about triple that of a sample incubated for 10 min. To identify the enzymatic product, we collected the suspected product fractions and used them for GC-mass analysis. The result shows that a major peak with retention period near 6.31 min in the GC chromatogram; the corresponding mass analysis yielded m/z 39, 53, 64, 81, 92, 110 (Fig. 3-7). This fragmentation pattern is virtually identical to that of a pure catechol standard. To analyze the catalytic activity of 1,2-dihydrogenase, we treated the reaction mixture containing catechol with the crude enzyme extract. The

resulting mixture was subjected to product analysis by ion chromatography as described in the method section. In Fig. 3-8, a new feature, as compared with both background chromatograms of catechol and crude enzyme sample, was observed at a retention period 2.2 min after the 2-min enzymatic catalysis of catechol. This feature was enhanced when *cis, cis*-muconic acid was spiked in the sample (chromatogram b) or when the duration of reaction was increased to 10 min. (chromatogram c). The eluent corresponding to the new peak area was collected and analyzed with electrospray ionization LC mass spectrometry (LC/MS) in a negative-charge mode and tandem mass spectrometry (MS/MS); the results are shown in Fig. 3-9 (a) and (b). The signal at m/z 163 is consistent with the molar mass of monosodium muconate. The tandem mass analysis exhibited the following signals with m/z 97, 137 and 163, which are identical to the fragment pattern of pure *cis, cis*-monosodium muconate. Based on the enzymatic product and activity analysis, we conclude that an ortho-fission pathway is involved in the degradation of phenol by this isolated strain.

Although both catechol and *cis, cis*-muconic acid are intermediates in the ortho-fission pathway of phenol degradation, we found that *C. albicans* TL3 only utilized catechol as a nutrient, unlike *Acinetobacter* sp. CNU961 (Jeong et al 1998) which can grow in the medium with either intermediate as a carbon source. Perhaps

C. albicans TL3 lacks a transport system for *cis, cis*-muconic acid so that no nutrient can be appropriately uptaken to support cell growth.

3.4.6 Application to the treatment of industrial effluent

To evaluate the prospective application of *C. albicans* TL3, we obtained a sample of waste water containing formaldehyde (65 mg/L; i.e. 2.17 mM) and phenol (6750 mg/L; i.e. 71.8 mM) from a local factory producing phenolic resin as a carbon source. *C. albicans* TL3 grew in the waste water diluted 5-fold. The cell growth and the residual concentrations of phenol and formaldehyde at various intervals are shown in Fig. 3-10. After incubation for six days with *C. albicans* TL3 at 30℃, phenol and formaldehyde were nearly completely degraded. The degradation of formaldehyde and phenol correlated well with the growth of *Candida albicans* TL3. Although some strains were reported to function as degraded of phenol and of trichloroethene (Folsom et al. 1990; Futamata et al. 2001; Chen et al. 2004), a microbe with a biodegrading power toward phenol and formaldehyde concurrently has been scarcely reported. *Candida albicans* TL3 is the first documented microbe that has a bio-degrading function for both phenol and formaldehyde. Being highly tolerant of phenol and having a large rate of degradation of phenol and a capacity to degrade phenol and formaldehyde directly in waste water are features that make *C.*

albicans TL3 particularly useful for treatment of waste water containing phenolic resin from its industrial sources. Chen et al. reported that the power to degrade phenol by *Candida tropicalis* was greatly improved with a simple cell-immobilization process (Chen et al. 2002). How to enhance the efficiency of degrading phenol by *Candida albicans* TL3 using immobilization technology will therefore be the subject of future work.

3.5 Conclusion

Here, we isolated a new phenol-degrading yeast strain — *C. albicans* TL3, which is obviously higher than other microorganisms in tolerance (up to 24 mM) and degradation rate of phenol. Exception phenol, interestingly, *C. albicans* TL3 can also degrade formaldehyde in waste water from a factory producing phenolic resin. Based on the analyses of enzymatic, chromatography and mass-spectrometry, we confirmed that this strain via an ortho-fission pathway to perform the degradation of phenol. The production of phenol hydroxylase and catechol 1,2-dioxygenase activities of *C. albicans* TL3 depended on cell growth temperature, phenol concentration and/or glucose. The maximum activity of phenol hydroxylase and catechol 1,2-dioxygenase were induced when this strain grew in phenol culture media at 22 mM and 10 mM, respectively. Alternatively, we suggest that synthesis of the phenol hydroxylase and catechol 1,2-dioxygenase would be modulated by one or more additional regulatory mechanisms such as catabolite repression.

3.6 References

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Table 3-1. Summary the capability of degradation of phenol by various yeasts.

Strain	Limit of phenol degradation (mM)	Reference
Trichosporon cutaneum	< 5.3	Neujahr and Varga 1970
Rhodotorula glutinis	5	Hirayama et al. 1994
Trichosporon dulcitum	15	Margesin et al. 2005
Basidiomycetous yeast	15	Margesin et al. 2005
Candida tropicalis	16	Bastos et al. 2000
Trichosporon cutaneum sp. LE3	18	Santos et al. 2001
Candida maltosa	18	Fialova et al. 2004
Candida tropicalis mutant	22	Chang et al. 1995
andida albicans TL3	24	this work

Table 3-2. Identification of the phenol-degradation isolated strain. This strain was dentified as *Candida albicans* by CBS in the Netherland.

Centraalbureau voor Schimmelcultures

Yeast Identification Service, Utrecht

Culture nr.: BN01-42 Identification nr. : **G02-09**

Growth temperature 25 °C

Morphology:

Pink colonies	–	Budding cells	+		
Lemon-shaped cells	–	Buds on stalks	–	Splitting cells	–
Filamentous	+	Pseudohyphae	+	Septate hyphae	+
Arthroconidia	–	Ballistoconidia	–	Symmetric ballistoconidia	–
Ascospores:	–				

Fermentation:

D-Glucose	+	Maltose	+	Lactose	–
D-Galactose	w	Sucrose	+	Raffinose	–

Growth on C compounds:

D-Glucose	+	Maltose	+	Glycerol	+
D-Galactose	+	α,α-Trehalose	+	Erythritol	–
L-Sorbose	–	Methyl α-D-glucoside	+	D-Glucitol	+
D-Glucosamine	+	Cellobiose	+	D-Mannitol	+
D-Ribose	–	Melibiose	–	myo-Inositol	–
D-Xylose	+	Lactose	–	2-Keto-D-gluconate	+
L-Arabinose	–	Raffinose	–	D-Gluconate	–
L-Rhamnose	–	Melezitose	+	D-Glucuronate	–
Sucrose	+			DL-Lactate	–

Growth on N compounds:

Nitrate	–	Ethylamine	+	L-Lysine	+
Cadaverine	+	D-glucosamine	–	D-tryptophane	+

Growth with:

0,01% Cycloheximide	+	Acetic acid production	–

Growth at: 37 °C +

Determined as: *Candida albicans*

nd=notdone,w=weak,d=delayed,v=variable

Table 3-3. Growth of *Candida albicans* TL3 on differents aromatic and related compounds（200ppm）after seven days in shake-flask.

Substrate	Growth（mg cell dry wt/ hr.L）
Benzene	×
Toluene	×
Anisol	×
p-Cresol	×
Catechol	3.85
Benzoic acid	×
Benzaldehyde	×
o-Cholorophenol	×
p-Carboxyphenol	×
m-Nitrophenol	×
2,4-Dintrophenol	×
cis-cis-Muconic acid	×

×:no growth

Table 3-4. Comparison of enzyme specific activity of *Candida albicans* TL3*

Source of carbon	Specific activity（unit/mg protein）	
	phenol hydroxylase	catechol-1,2-dioxygenase
glucose, 0.2 %	0	0
phenol, 5 mM	0.025±0.0008	0.270±0.01
phenol, 10 mM	0.044±0.0021	0.734±0.037
phenol, 15 mM	0.083±0.0046	0.217±0.019
phenol, 15 mM ＋glucose, 0.1%	0.057±0.0027	0.174±0.008
phenol, 15 mM ＋glucose, 0.2%	0.028±0.0022	0.149±0.012
phenol, 15 mM ＋glucose, 0.4%	0.014±0.0018	0.128±0.007
phenol, 20 mM	0.115±0.0063	0.107±0.009
phenol, 22 mM	0.156±0.011	0.101±0.005
phenol, 24 mM	0.129±0.0045	0.087±0.006

* Growth conditions: YNB medium (0.67 %) containing phenol and/or glucose, at 30℃. Data are expressed as mean ± standard deviation (*n*=3).

Table 3-5. Effect of temperature on specific activity of phenol hydroxylase and catechol-1,2-dioxygenase from *Candida albicans* TL3.*

Growth temperature (℃)	specific activity（unit/mg protein）	
	phenol hydroxylase	catechol-1,2-dioxygenase
25	0.087±0.0041	0.275±0.012
30	0.083±0.0046	0.217±0.019
35	0.064±0.0037	0.158±0.007
40	0.061±0.002	0.100±0.003

* The microbe was grown with YNB medium (0.67 %) containing phenol (15 mM).

Data are expressed as mean ± standard deviation (*n*=3).

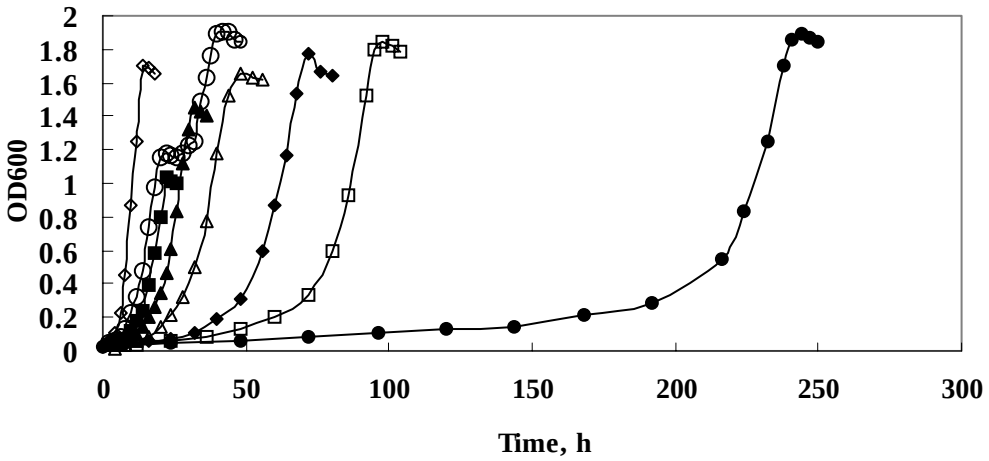


Figure 3-1. Time-course profiles of cell growth of *Candida albicans* TL3. The cells were incubated at 30℃ with YNB medium (0.67 %) containing phenol at various concentrations: 5 mM (■); 10 mM (▲); 15 mM (△); 20 mM (◆); 22 mM (□); 24mM (●), 0.2 % glucose (◇), and the mixture of 15-mM phenol + 0.2% glucose (○). Each data point represents the mean of triplicate independent measurements. Data shown are the mean of triplicate experiments with standard deviation within 10%.

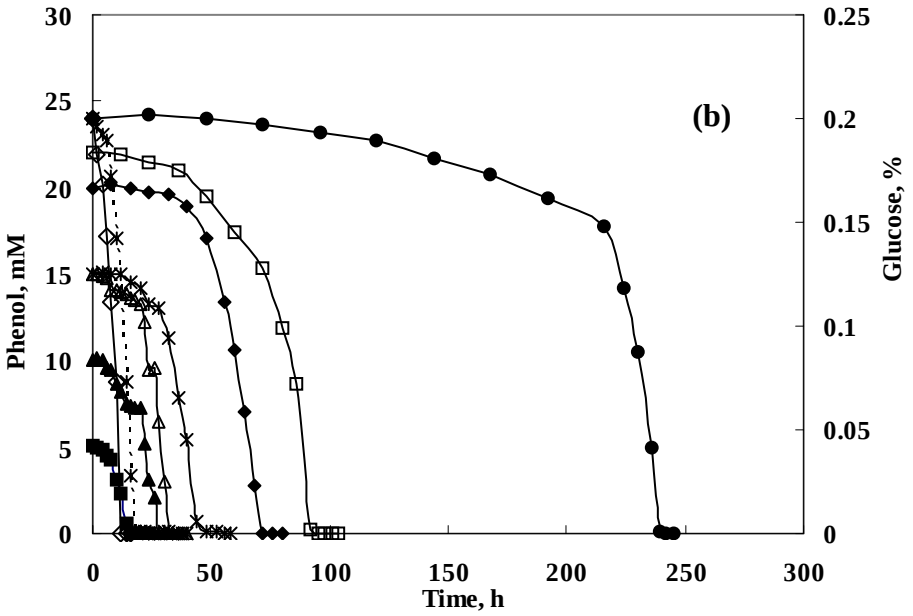


Figure 3-2. Consumption of phenol and glucose of *Candida albicans* TL3. The cells were incubated at 30℃ with YNB medium (0.67 %) containing phenol at various concentrations: 5 mM (■); 10 mM (▲); 15 mM (△); 20 mM (◆); 22 mM (□); 24 mM (●), 0.2 % glucose (◇), and the mixture of 15-mM phenol + 0.2% glucose (○), and the residual concentrations of phenol (×) and glucose (-✳-) in the mixture medium. Each data point represents the mean of triplicate independent measurements. Data shown are the mean of triplicate experiments with standard deviation within 10%.

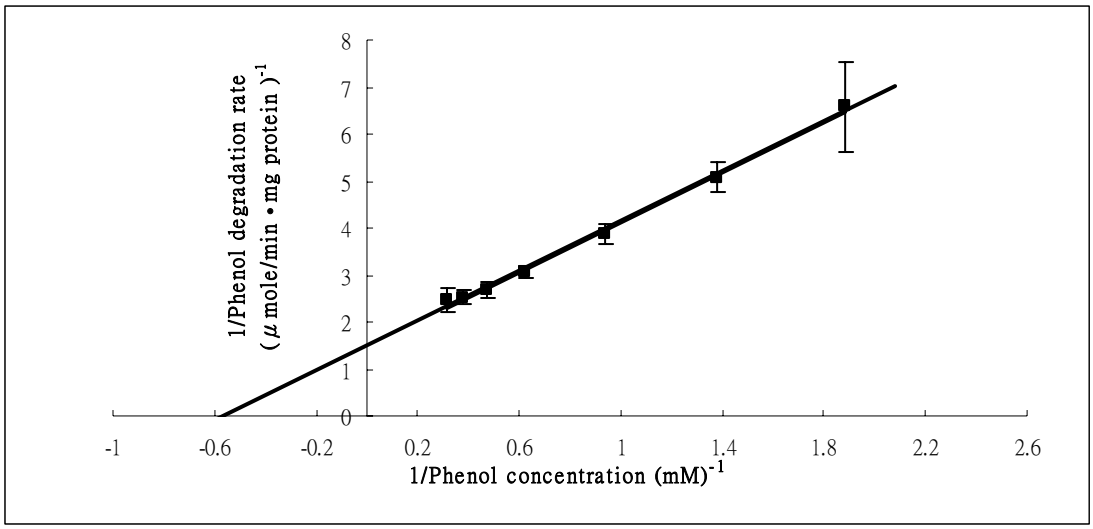


Figure 3-3. The kinetic parameters of phenol biodegradation catalyzed by *C. albicans* TL3. Each data point represents the mean of triplicate independent measurements.

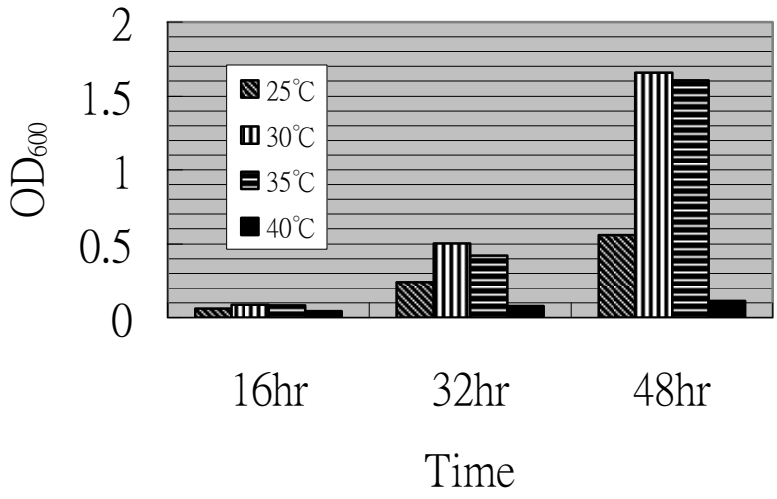


Figure 3-4. Temperature effect on the growth of *Candida albicans* TL3. The cell was grown on 15 mM phenol at the indicated temperatures.

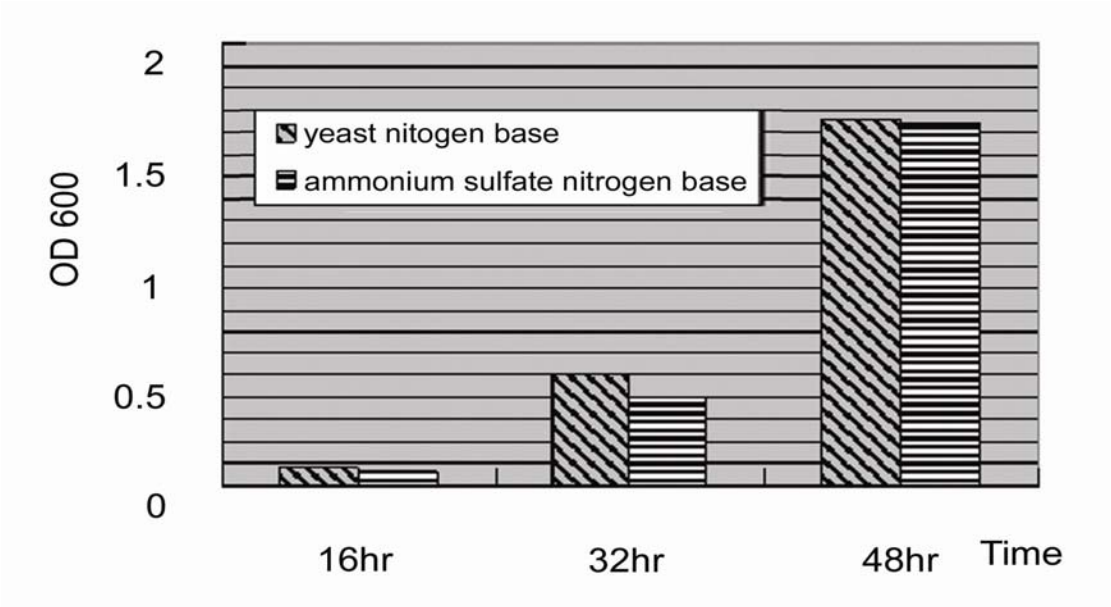


Figure 3-5. Comparison of the growth of *Candida albicans* TL3 with different nitrogen bases. The cell was grown on phenol (15 mM) containing nitrogen base at 30℃.

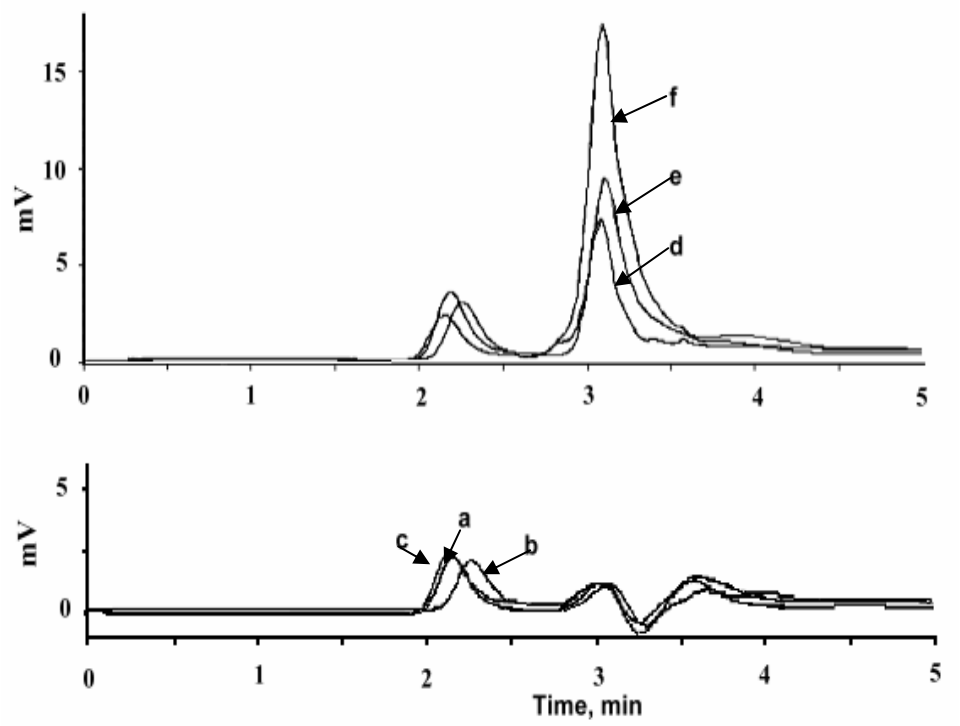


Figure 3-6. HPLC analysis of the product of phenol is catalyzed by crude enzyme extract. Reactions were terminated at 10 or 25 min on addition of excess methanol. Samples taken from the mixtures were then centrifuged to remove the precipitant before applying to a column. The resulting supernatants containing varied compositions were analyzed: (a) without addition of NADPH and phenol; (b) with phenol; (c) with NADPH; (d) 10-min reaction with both NADPH and phenol; (e) with spiking 0.02-mM catechol in the sample (d); (f) 25-min reaction with both NADPH and phenol. The extract medium of crude enzyme as described in the text.

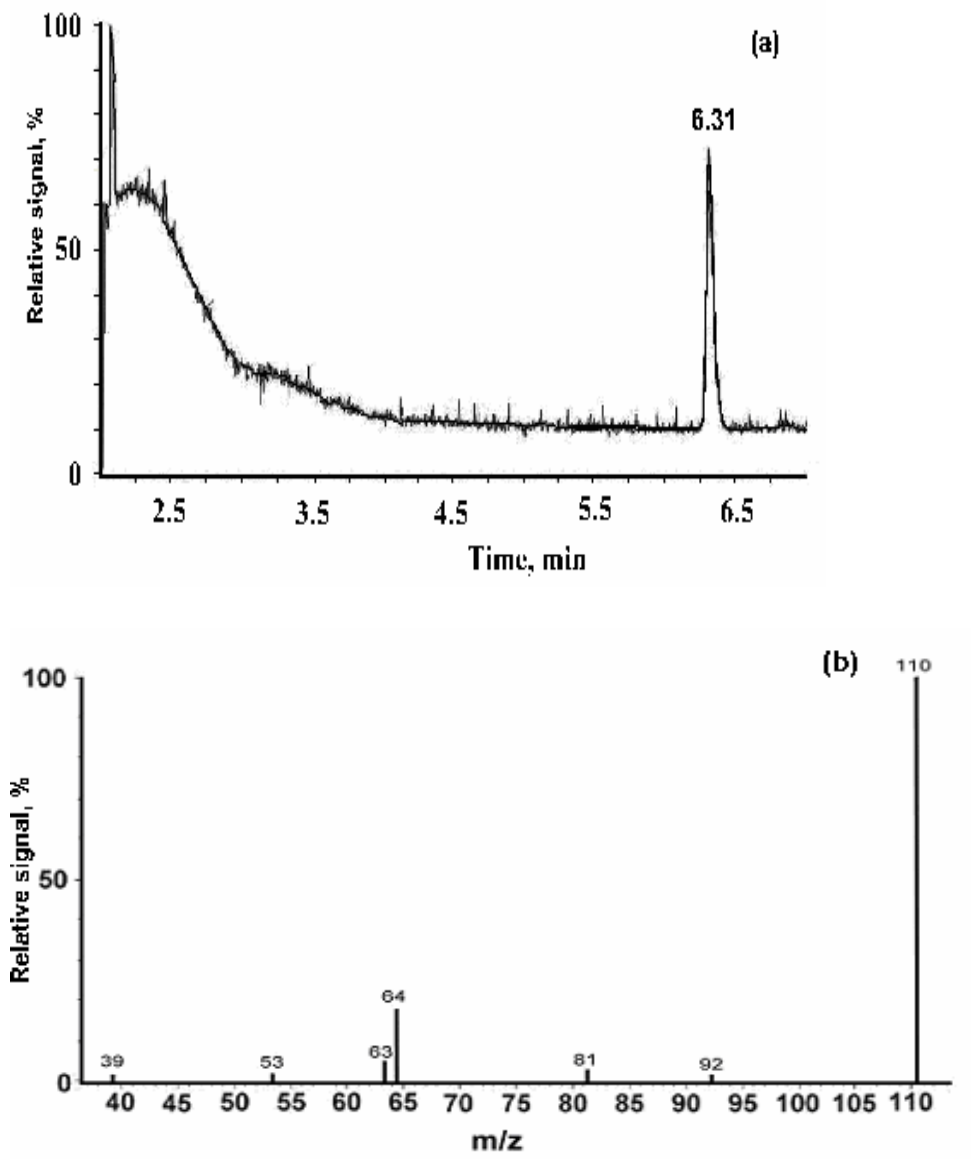


Figure 3-7. GC-mass analysis of the product of phenol is catalyzed by crude enzyme extract. The sample containing catechol, the anticipated product of phenol hydroxylase-catalyzed reaction, was analyzed and confirmed by (a) GC chromatography integrated with (b) electron ionization mass spectrometry (EI/MS). The feature at m/z 110 corresponds to the molecular ion of catechol (M^+). All fragments shown in (b) are consistent with those of standard catechol.

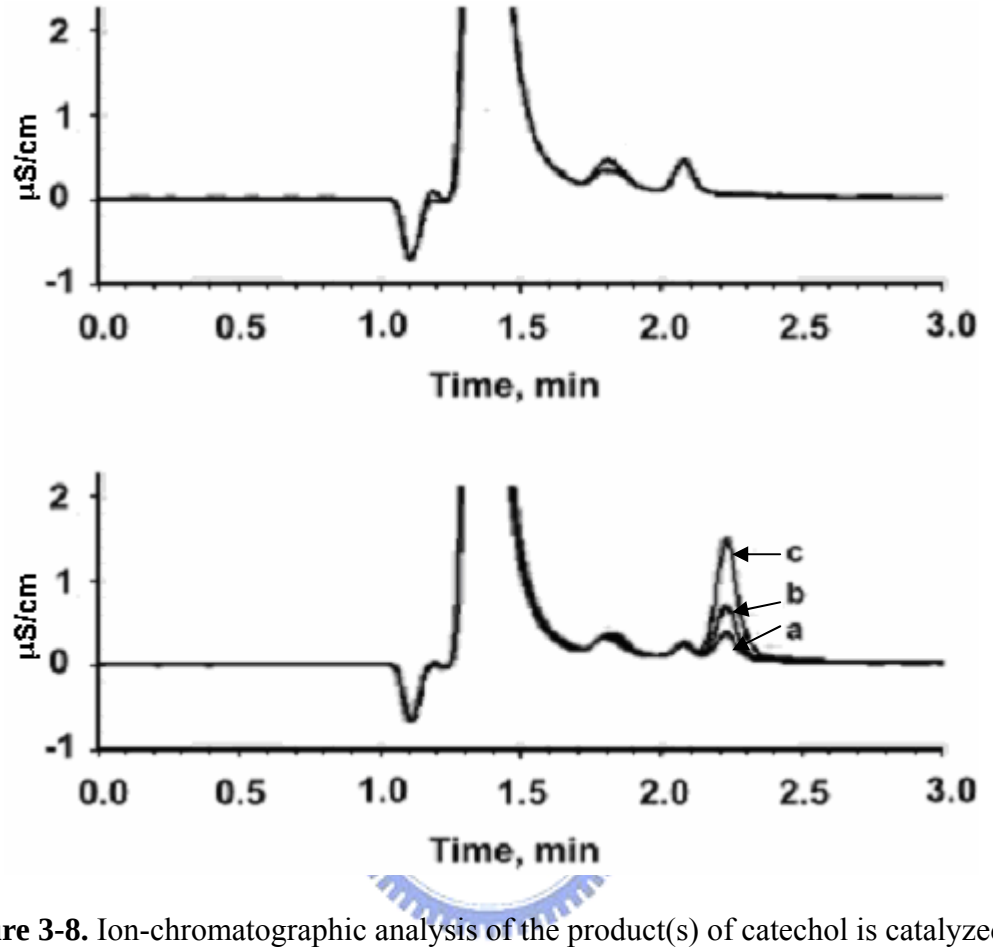


Figure 3-8. Ion-chromatographic analysis of the product(s) of catechol is catalyzed by crude enzyme extract. Reactions were stopped at 2 min on addition of excess methanol. Samples taken from the mixtures were then centrifuged to remove the precipitant before applying to a column. The upper figure shows the overlay of two chromatograms without addition of either the crude enzyme extract or catechol. The lower figure contains the overlay of chromatograms with sample from (a) 2-min reaction mixture; (b) 2-min reaction mixture with spiking of 0.015 mM *cis, cis*-muconic acid; (c) 10-min reaction mixture.

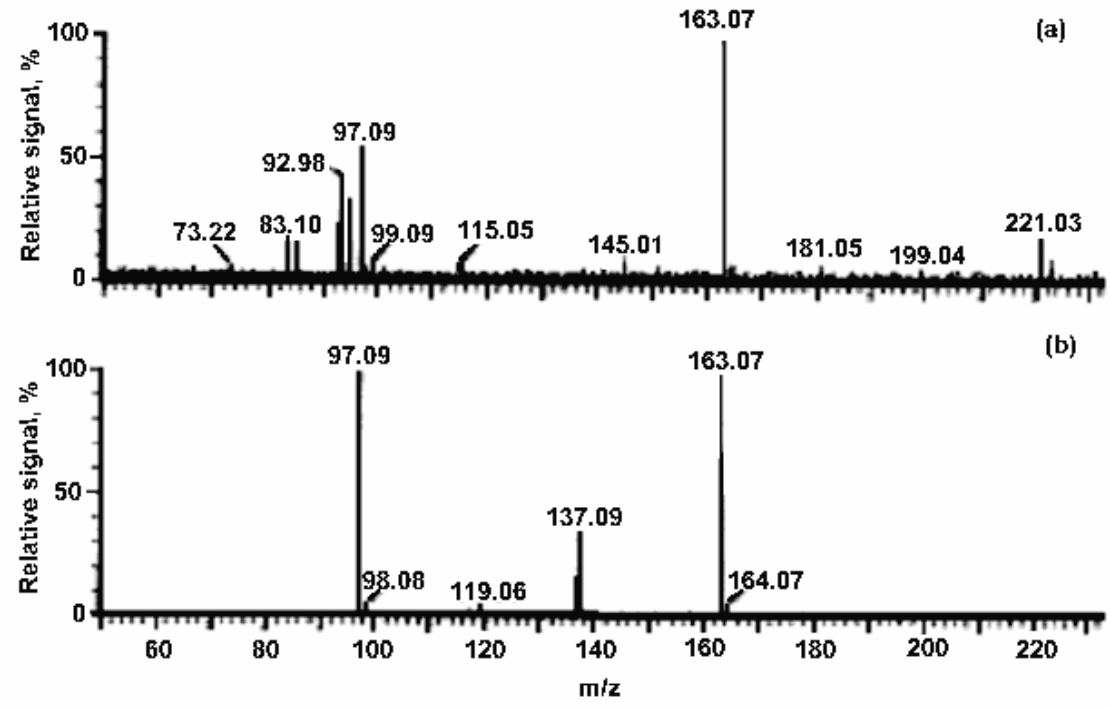


Figure 3-9. Electrospray ionization mass analysis (ESI) of the product of catechol is catalyzed by crude enzyme extract. The sample containing monosodium muconic acid (mw.164), the anticipated product of catechol 1,2-dioxygenase-catalyzed reaction, was analyzed by ESI/MS (a) and confirmed by ESI/MS/MS (b). The feature at m/z 163 corresponds to monosodium muconate (M^-). The pattern of fragments shown in (b) is identical to that of standard monosodium muconate.

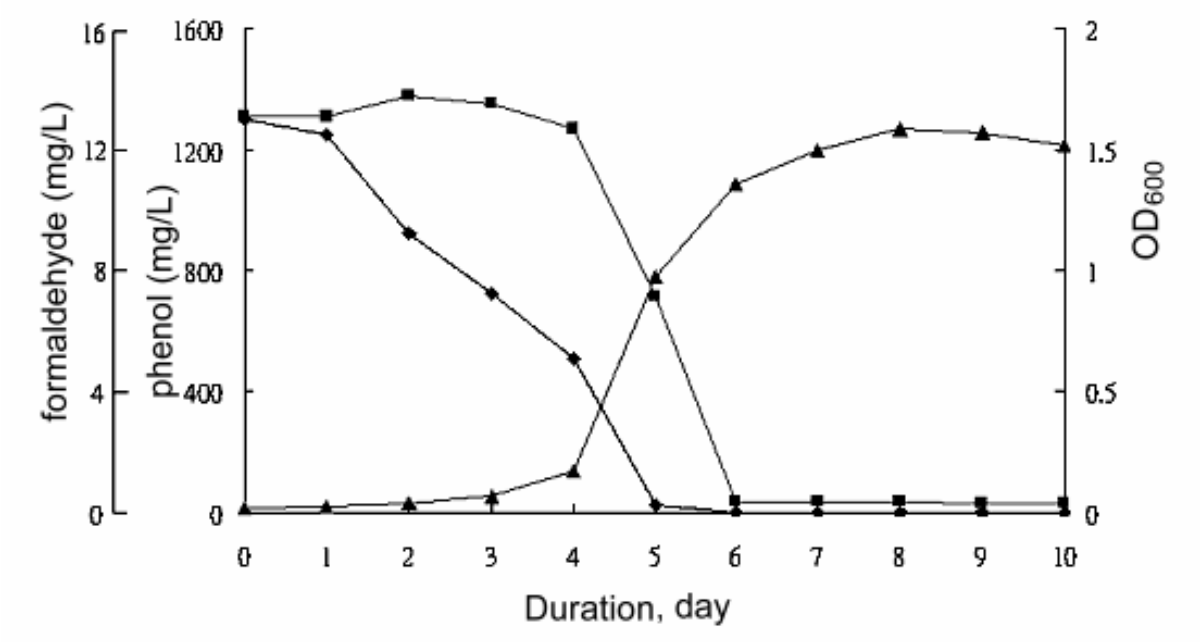


Figure 3-10. Growth (▲) and phenol (■) and formaldehyde (◆) consumption of *Candida albicans* TL3 was cultured on waste water as a sole carbon source. At the beginning, *C. albicans* TL3 culture (0.3 mL, OD_{600} = 1.5) at a stationary phase was added to waste water (49.7 mL) containing phenol (14.5 mM) and formaldehyde (0.44 mM). Each data point represents the mean of triplicate independent measurements. Data are expressed as mean ± standard deviation (*n*=3).

Chapter 4

Purification and characterization of a catechol 1,2-dioxygenase from a phenol degrading *Candida albicans* TL3

4.1 Abstract

A novel catechol 1,2-dioxygenase (1,2-CTD) was induced from a eukaryotic *Candida albicans* TL3 strain that possesses high tolerance for phenol and strong phenol degrading activity. The 1,2-CTD was purified via ammonium sulfate precipitation, Sephadex G-75 gel filtration, and HiTrap Q Sepharose column chromatography. The enzyme was purified to homogeneity and found to be a homodimer with a subunit molecular weight of 32,000. Each subunit contained one iron. Optima for temperature and pH are 25 °C and pH 8.0, respectively. Substrate analysis showed that the purified enzyme is a type I catechol 1,2-dioxygenase and has higher catalytic activity toward catechol than 4-methylcatechol. The k_{cat} and K_m values of 1,2-CTD for catechol were 28 s^{-1} and 9.3 μM, respectively. When 4-methylcatechol was used as substrate, the k_{cat} value (5.6 s^{-1}) was only 20% of that of catechol and the K_m value (21.5 μM) was about 2.3-fold larger than that of catechol.

Keywords: catechol 1,2-dioxygenase, *Candida albicans* TL3

4.2 Introduction

Catechols are formed during biodegradation of a variety of aromatic compounds by aerobic microorganisms. The aromatic rings of catechols may be cleaved by catechol 1,2-dioxygenases (1,2-CTDs), hydroxyquinol 1,2-dioxygenases(1,2-HQDs) (type I catechols 1,2-dioxygenase) (Nakai et al. 1990; Eck et al. 1991; Murakami et al. 1997; Briganti et al. 1997; Shen et al. 2004), chlorocatechol 1,2-dioxygenases (1,2-ClCTDs) (type II catechols 1,2-dioxygenase) (Broderick et al. 1991; Van der Meer et al. 1993; Maltseva et al. 1994) and protocatechuate 3,4-dioxygenases (3,4-PCDs) via an orth-cleavage pathway to form cis,cis-muconate or its derivates or by catechols 2,3-dioxygenases via a meta-cleavage pathway to form 2-hydroxymuconic semialdehyde or its derivates. These metabolites are degraded in the tricarboxylic acid cycle (Ngai, et al. 1990; Chen and Lovell 1990; Aoki et al. 1990). Among the catechols dioxygenases, 1,2-CTDs have been well characterized in terms of their biochemical properties (Aoki et al. 1984; Murakami et al. 1997; Briganti et al. 1997; Strachan et al. 1998; Strachan et al. 1998; Ridder et al. 1998).

In general, catechol 1,2-CTDs are dimeric proteins with identical or similar subunits, which have molecular weights of 30,500–34,000 Da per subunit (Eck and Bettler 1991; Neidle et al. 1988) and possess one or two iron molecule in the dimeric form (Neidle and Ornston 1986, Briganti et al. 1997). 1,2-CTDs are specific for

catechols, alkylated catechols.

Although there are many reports concerning 1,2-CTDs, they are almost from bacteria. There are few reports on 1,2-CTDs from eukaryotic bacteria. Previously, we isolated a strain of yeast, *Candida albicans* TL3, which uses phenol and/or formaldehyde as its energy source, from soil at a petrochemical factory in Taiwan. It has high tolerance for phenol (up to 24 mM) and catalyzes phenol degradation through the ortho-cleavage pathway (Tsai et al. 2005). We thus attempted to purify the1,2-CTD of *Candida albicans* TL3 and characterize it compared with the 1,2-CTDs of bacteria strains. The present paper describes the purification and biochemical properties of the 1,2-CTD from *C. albicans* TL3 that grown in the presence of 10 mM phenol.

4.3 Experimental

4.3.1 Cell culture

Candida albicans TL3, which had previously been isolated from soil at a petrochemical plant by our group, was cultured at 30 °C with an initial optical density at 600 nm (OD_{600}) of 0.01 in a flask containing YNB medium (0.67% w/v) and 10 mM phenol (Tsai et al. 2005). Cells were harvested when the growth of *C. albicans* TL3 approached the stationary phase (OD_{600} of approximately 1.4). After centrifugation, the pelleted cells were washed twice with distilled water.

4.3.2 Preparation of crude extract and enzyme purification

Harvested cells were suspended in 50 mM Tris-HCl buffer (pH 8.3) containing 5 mM β-mercaptoethanol and then disrupted by sonication (60% amplitude and two pulses per second) with an ultrasonic processor (VCX-750, Sonics, USA). After centrifugation (13,700 × *g* for 30 min at 4 °C), the supernatant was collected and used as a crude enzyme extract. Enzyme purification was performed at 4 °C as follows:

Step 1: Ammonium sulfate fractionation

Ammonium sulfate was added to the crude extract to result in 50−70% saturation. The precipitated protein was obtained by centrifugation at 22,860 × *g* for 30 min at 4 °C. The pellet was resuspended in 3 ml Tris-HCl buffer (50 mM, pH 8.3).

Step 2: Sephadex G-75 fractionation

A column (2 cm × 80 cm) packed with Sephadex G-75 (Sigma) was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.3). The crude protein solution was loaded onto the Sephadex G-75 column and eluted with 50 mM Tris-HCl buffer (pH 8.3) at a flow rate of 0.1 ml/min. Sequential 1 ml aliquots of eluent were collected and tested for 1,2-CTD activity. Fractions that exhibited enzyme activity were pooled and concentrated by ultrafiltration on an Amicon ultracentrifugal filter (MWCO:10000, Millipore Co., USA).

Step 3: Chromatography on Q Sepharose

The concentrated protein solution obtained after completion of Step 2 was purified further using a HiTrap Q Sepharose column (5 ml × 2; Pharmacia) and FPLC system. Proteins were eluted with a linear gradient from 0 M to 0.2 M of $(NH_4)_2SO_4$ in Tris-HCl buffer (50 mM, pH 8.3) at a flow rate of 0.5 ml/min. Each 1 ml fraction was collected and used for the activity assay.

4.3.3 Determination of protein concentration

Protein concentration was determined using bovine serum albumin as a standard by the method of Bradford (Bradford 1976), and Scopes method (Scopes 1974) was also used to determine protein concentration in the analysis of iron content.

4.3.4 Determination of molecular mass

The molecular weight of the subunit of 1,2-CTD was determined using 12.5%

SDS-PAGE as described by Laemmli (Laemmli 1970). Coomassie Brilliant Blue R250 was used as a protein stain. The molecular weight of the native protein was determined using gel filtration on a Sephadex G-75 column and a series of standard proteins.

A more precise estimate of subunit molecular weight was obtained using electrospray ionization mass spectrometry (ESI-MS). Mass spectra were recorded with a quadrupole time-of-flight mass spectrometer (Q-TOF, Micromass, UK). The quadrupole mass analyzer scanned mass-to-charge ratios (m/z) from 100 to 2500 units with a scan step of two seconds and an interscan of 0.1 seconds per step. In the ESI-MS experiment, we used the quadrupole scan mode under a capillary needle at 3 kV, a source block temperature of 80 °C and a desolvation temperature of 150 °C. Between five and 10 µg of protein in desalted form were used for MS.

4.3.5 Enzyme activity assays

1,2-CTD activity was determined from the rate of accumulation of *cis*,*cis*-muconic acid (increase in absorbance at 260 nm) (Varga and Neujahr 1970). One unit of 1,2-CTD was defined as the amount of enzyme that catalyzes the formation of 1 µmol *cis,cis*-muconic acid or its derivatives per minute. The assay mixture contained 2 µl enzyme solution per milliliter and 998 µl 50 mM Tris-HCl buffer, pH 8.3, per ml. The Tris-HCl buffer contained 5 mM β-mercaptoethanol, 20

μM $FeSO_4$, and 0.1 mM catechol.

The substrate specificity, optimal temperature, and optimal pH for enzyme activity of purified 1,2-CTD were determined using catechol analogues as substrate instead of catechol, at various temperatures and pH values. The increase in absorbance was measured at 260 nm for products from all substrates except hydroxyquinol and protocatechuate, for which wavelengths of 245 nm and 270 nm were used, respectively (Bull and Ballou 1981; Strachan et al. 1998).

To investigate the thermal and pH stabilities of the enzyme and the effects of various compounds on its enzyme activity, it was preincubated for 30 min at various temperatures, pH values, metal ions, and other compounds. The residual enzyme activities were measured by adding catechol and carrying out the enzyme assay at pH 8.3 and 25 °C.

4.3.6 Kinetic measurements

The initial velocity of catechol-1,2-dioxygenase from *C. albicans* TL3 was estimated at 25 °C and pH 8.3 and expressed as a function of catechol (1–150 μM) and 4-methylcatechol (2.5–300 μM) at enzyme concentrations of 0.072 μg/ml and 0.43 μg/ml, respectively. Estimates of kinetic parameters were determined graphically from double reciprocal plots.

4.3.7 Iron analysis

The purified enzyme was nitrated in 6N HNO_3 and then the iron content of the protein was determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500a, USA). All glassware was acid-washed to avoid contamination with iron.

4.4 Results and Discussion

4.4.1 Purification of 1,2-CTD

Purification of 1,2-CTD from cell-free extracts of *C. albicans* TL3 was performed using ammonium sulfate precipitation and Sephadex G-75 gel filtration (Fig. 4-1) followed by HiTrap Q Sepharose column chromatography (Fig. 4-2) as described in the experimental methods. Yields for each step of the purification process are summarized in Table 4-1. Purification enhanced the purity of 1,2-CTD 40-fold and produced a product with a specific activity of 63.0 units per mg protein and a yield of 33%. Gel-filtration analysis using a Sephadex G-75 column showed that the purified enzyme eluted at a position corresponding to a molecular mass of about 64 kDa (Fig. 4-3). After SDS-PAGE, the purified enzyme appeared as a single band with a molecular mass of 31 kDa (Fig. 4-4), suggesting that the enzyme is a dimeric protein. The molecular mass of the subunit of 1,2-CTD was estimated by ESI-MS to be 31,994 ± 2 Da (Fig. 4-5), which is consistent with the results of the SDS-PAGE analysis. The dimeric nature of this eukaryotic 1,2-CTD is similar to that of prokaryotic bacteria, which have molecular weights of 30,500–34,000 Da per subunit (Eck and Bettler 1991; Neidle et al. 1988).

4.4.2 Characterization of 1,2-CTD

The 1,2-CTD of *C. albicans* TL3 showed considerable activity towards catechol and 4-methylcatechol (18% of catechol), but no significant activity towards other catechol derivatives such as 3-methycatechol, 4-chlorocatechol, 4-carboxycatechol,

and hydroxyquinol (Table 4-2). ESI tandem mass spectrometric analysis showed that the products of the reaction between 1,2-CTD and catechol or 4-methylcatechol were disodium muconate and 3-methyl-disodium muconate, respectively (Fig. 4-6). Because of its substrate specificity, we suggest that the 1,2-CTD of *C. albicans* TL3 possesses characteristics similar to those of *Acinetobacter* sp. (Caposio et al. 2002; Kim et al. 2003), *Pseudomonas arvilla* C-1 (Nakai et al. 1990), and *Frateuria* sp. ANA-18 (Aoki et al. 1984), which are classified as type I 1,2-CTDs. The k_{cat} and K_m values of 1,2-CTD for catechol were 28 s^{-1} and 9.3 μM, respectively. When 4-methylcatechol was used as substrate, the k_{cat} value (5.6 s^{-1}) was only 20% of that of catechol and the K_m value (21.5 μM) was about 2.3-fold larger than that of catechol (Fig. 4-7 and Fig. 4-8). This result showed that the enzyme has higher affinity and catalytic activity for catechol than 4-methylcatechol.

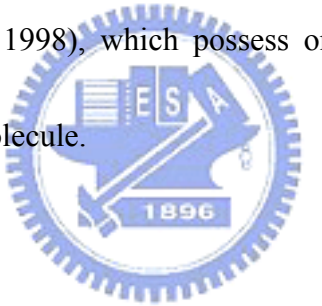
The optimal temperature and pH of 1,2-CTD from *C. albicans* TL3 was 25 °C and pH 8.0, respectively (Fig. 4-9 and Fig. 4-10). The optimal pH of this enzyme is similar to that of 1,2-CTDs that have been isolated from *Pseudomonas* sp. (Nakai et al. 1988; Briganti et al. 1997) and *Acinetobacter* sp. (Kim et al. 2003), but lower than that isolated from *Rhizobium leguminosarum* (Chen and Lovell 1990), *Rhizobium trifolii* (Chen et al. 1985), and *Rhodococcus rhodochrous* (Strachan et al. 1998), which are optimally active at pH 9.0−9.5. The enzyme was found to be stable when

it was kept at temperature lower than 40 °C (Fig. 4-11). The purified enzyme was stable (maintaining > 85% activity) for at least 30 min at the pH range of 7.0 to 9.0, whereas the stability of enzyme greatly decreased in the pH condition out of this range (Fig. 4-12). The biochemical properties of 1,2-CTD from *C. albicans* TL3 are summarized in Table 4-3.

The effects of metal salts, chelating agents, and sulfhydryl agents on the activity of this enzyme were investigated. The results are shown in Table 4-4. The activity of this enzyme for catechol was minimally affected by $FeSO_4$, $FeCl_3$, $CuSO_4$, $CoCl_2$, $MnSO_4$ or EDTA at concentrations of up to 0.1 mM, whereas the addition of 0.1 mM $AgNO_3$, CuCl, $HgCl_2$ or $PbSO_4$ inhibited the enzymatic reaction by 41%–96%. Interestingly, the presence of β-mercaptoethanol (5 mM) enhanced activity by 254%. The presence of Ag^+ and β-mercaptoethanol inhibited and enhanced obviously this enzyme activity, respectivity, it seems that sulfhydryl group of cysteine residue(s) play an important role in 1,2-CTD activity from *C. albicans* TL3, which agree well with *Acinetobacter* sp. KS-1 (Kim et al. 2003) and *Frateuria* species ANA-18 (Aoki et al. 1984).

The metal content of 1,2-CTD was determined by inductively coupled plasma-mass spectrometry (ICP-MS). The concentration of iron in the purified

enzyme was estimated to be 0.0243 (±0.0012) nmole per 0.0127 (±0.0005) nmole (determined by Bradford method) or 0.0133 (±0.0007) nmole (determined by Scopes method) of the protein. These results suggest that the 1,2-CTD of *C. albicans* TL3 contains one iron per subunit. The iron content of this 1,2-CTD is similar to that of *Acinetobacter calcoaceticus* (Neidle and Ornston 1986) and *Rhizobium trifolii* TA1 (Chen et al. 1985), but differs from that of *Brevibacterium fuscum* (Nakazawa 1963), *Pseudomonas* sp. (Nakai et al. 1988; Briganti et al. 1997), *Rhizobium leguminosarum* biovar viceae USDA2370 (Chen and Lovell 1990), and *Rhodococcus rhodochrous* NCIM13259 (Strachan et al. 1998), which possess only one iron molecule in the dimeric form of the protein molecule.



4.5 Conclusion

To the best of our knowledge, this is the first report on the purification and characterization of 1,2-CTD from eukaryotic cell(s). The 1,2-CTD from *C. albicans* TL3 had substrate specificity similar to that of type I 1,2-CTD from bacteria. From the result of metal ions and β-mercaptoethanol effect on enzyme activity, which is presumably sulfhydryl group of cysteine(s) may be involved in this enzyme activity. In addition, this enzyme displayed a high level of homology with bacteria 1,2-CTDs in molecular mass, iron content, temperature, pH and metal ion sensibilities.

4.6 Referances

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Table 4-1. Purification of catechol-1,2-dioxygenase from *C. albicans* TL3.

Step	Fraction	Volume (ml)	Proteins (mg/ml)	Specific activity (units/mg)	Purification fold	Yield(%) (enzyme activity)
1	Crude extract	34	2.89	1.57	1	100
2	$(NH_4)_2SO_4$ ppt, 50-70%	3	3.31	12.7	8.1	81.5
3	Eluate, Sephadex G-75 column	5	0.52	25.6	16.3	42.6
4	Eluate, HiTrap Q Sepharose column	1.2	0.68	63.0	40.1	33.1

Table 4-2. Substrate specificity of 1,2-CTD from *C. albicans* TL3

Substrate	Relative enzyme activity (%)
Catechol	100
3-Methylcatechol	0
4-Methylcatechol	18
4-Chlorocatechol	0
4-Carboxycatechol	0
Hydroxyquinol	0
Protocatechuate	0
2,3-dihydroxybenzoic acid	0
3,4-dihydroxybenzaldehyde	0
3,4-dihydroxybenzylamine	0

Table 4-3. The properties of 1,2-CTD from *C. albicans* TL3.

Molecular weight (Da)	SDS-PAGE	31,000
	ESI-MS	31,994
	Gel filtration	64,000
Iron content (mol /mol enzyme)		2
Optimum pH		8.0
Optimum temperature		25 °C
Stability of pH		7.0~9.0
Stability of temperature		below 40 °C
K_m for catechol, 4-methylcatechol		9.3 μM, 21.6 μM
k_{cat} for catechol, 4-methylcatechol		28 s^{-1}, 5.6 s^{-1}

Table 4-4. Effects of some metal ions and compounds on the activity of 1,2-CTD from *C. albicans* TL3 for catechol.

Substance	Remaining activity (%)	
	0.01 mM	0.1 mM
None	100	100
$AgNO_3$	7 ± 2	4 ± 2
CuCl	72 ± 4	59 ± 1
$FeSO_4$	109 ± 10	95 ± 5
$FeCl_3$	115 ± 9	86 ± 4
$MnSO_4$	94 ± 5	113 ± 6
$CuSO_4$	95 ± 3	93 ± 2
$CoCl_2$	97 ± 12	92 ± 5
$HgCl_2$	85 ± 1	36 ± 2
$PbSO_4$	81 ± 3	25 ± 4
EDTA	99 ± 7	101 ± 9
MSH*	192 ± 20	254 ± 28

* The activity of the enzyme was increased to 192% and 254% of that of the intact enzymeby the addition of 1 mM and 5 mM β-mercaptoethanol, respectively.

Data are expressed as the mean ± standard deviation (n = 3).

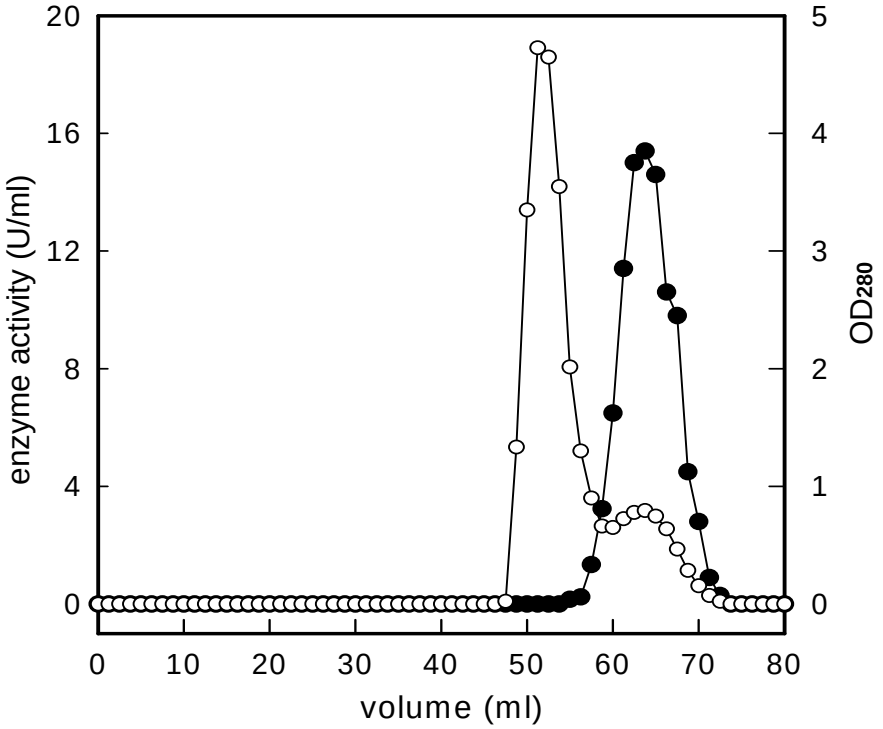

Figure 4-1. Separation of catechol 1,2-dioxygenase from 50-70% $(NH_4)_2SO_4$ ppt on a G-75column (2x80 cm). Catechol 1,2-dioxygenase (●), absorbance at 280nm (○).

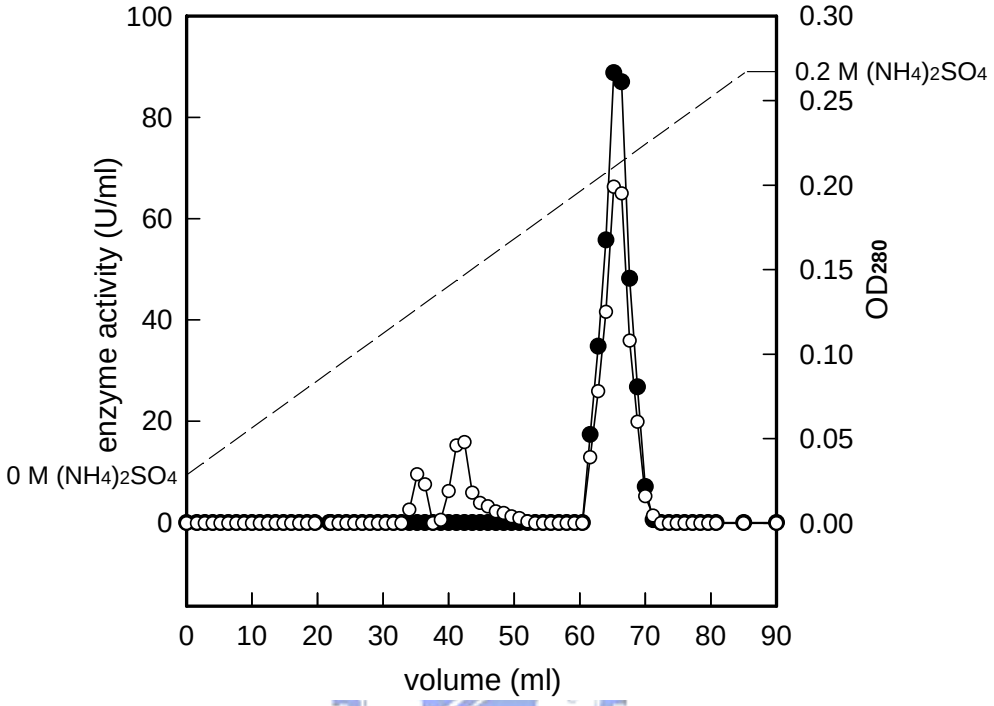

Figure 4-2. Separation of catechol 1,2-dioxygenase from the catechol 1,2-dioxygenase-containing fractions of G-75 column on a Q-sephadex column. Catechol 1,2-dioxygenase (●), absorbance at 280nm (○).

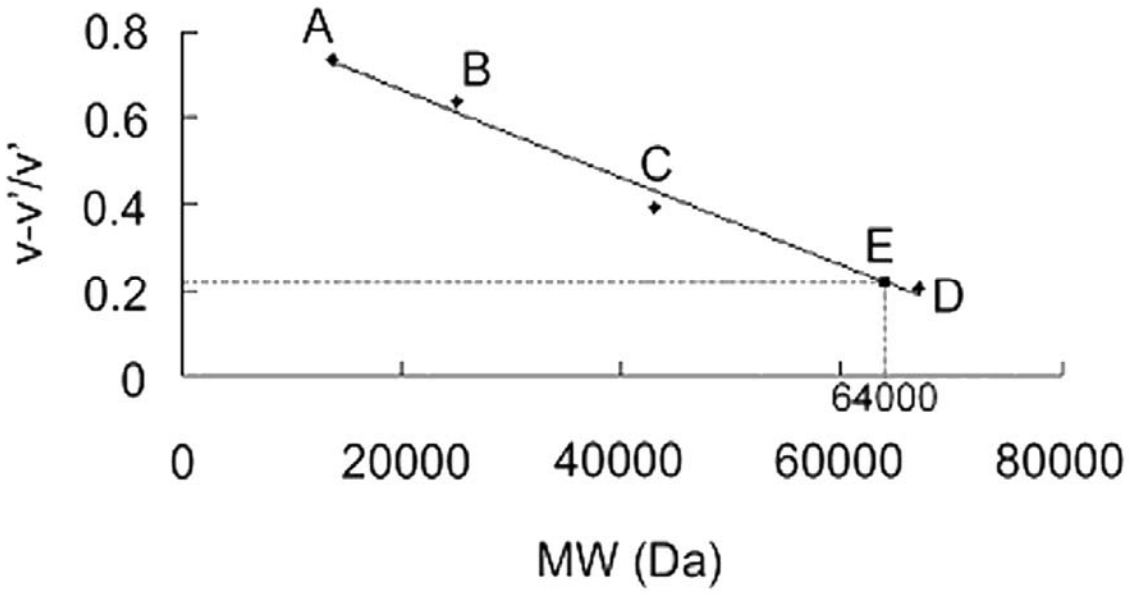

Figure 4-3. Native molecular mass determination of 1,2-CTD from *C. albicans* TL3 by G-75 column Chromatography. A, ribonuclease A (13.7 KDa); B, chymotrypsin (25 KDa); C, ovalbumin (43 KDa); D, albumin (67 KDa); E, 1,2-CTD (64 KDa). V and V′ are elution volume for sample protein and blue dextran (2000 KDa), respectively.

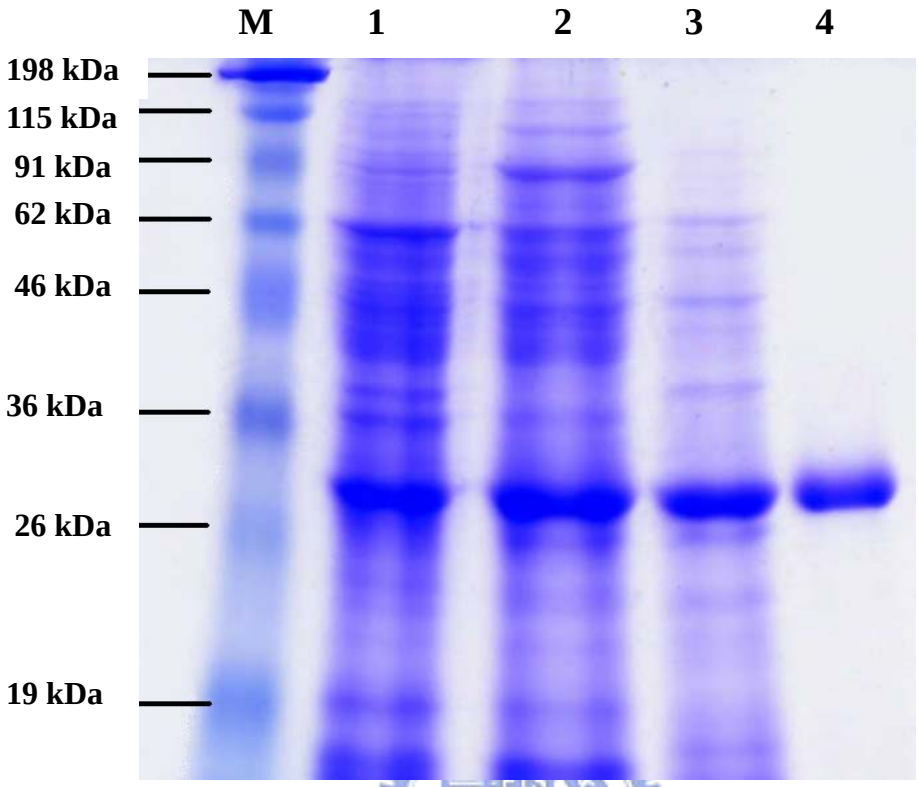

Figure 4-4. SDS-PAGE analysis of 1,2-CTD from *C. albicans* TL3 in various steps of purification. Lane M, protein markers; Lane 1, crude cell extract, 170 μg protein; Lane 2, 50%–70% $(NH_4)_2SO_4$ precipitation, 26 μg protein; Lane 3, after chromatography on a G-75 column, 4 μg protein; Lane 4, after chromatography on a HiTrap Q Sepharose column, 2.5 μg protein.

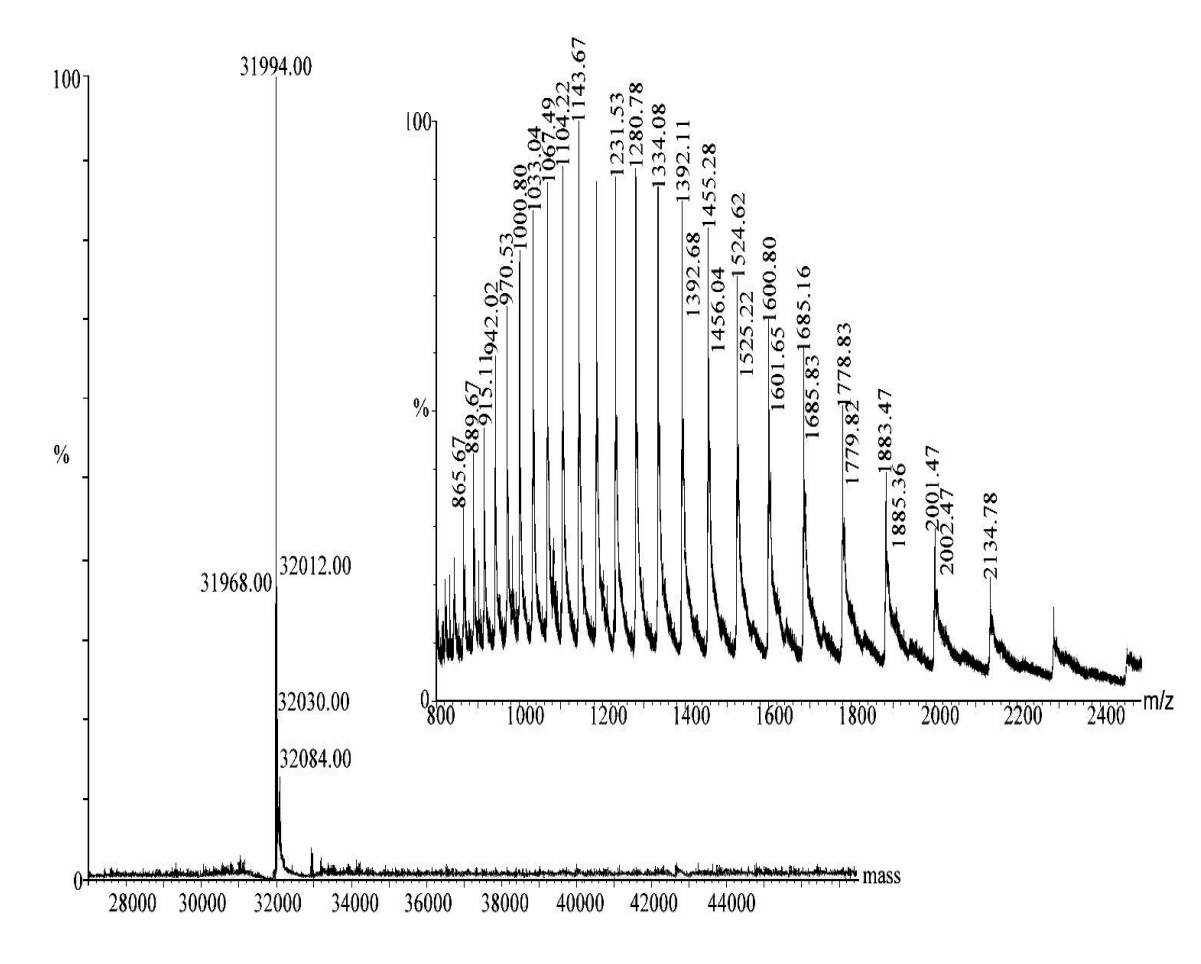

Figure 4-5. The mass spectrum of the purified 1,2-CTD from *C. albicans* TL3 (*inset*) and the deconvolution of the spectrum to give a molar mass of 31,994 atomic mass units.

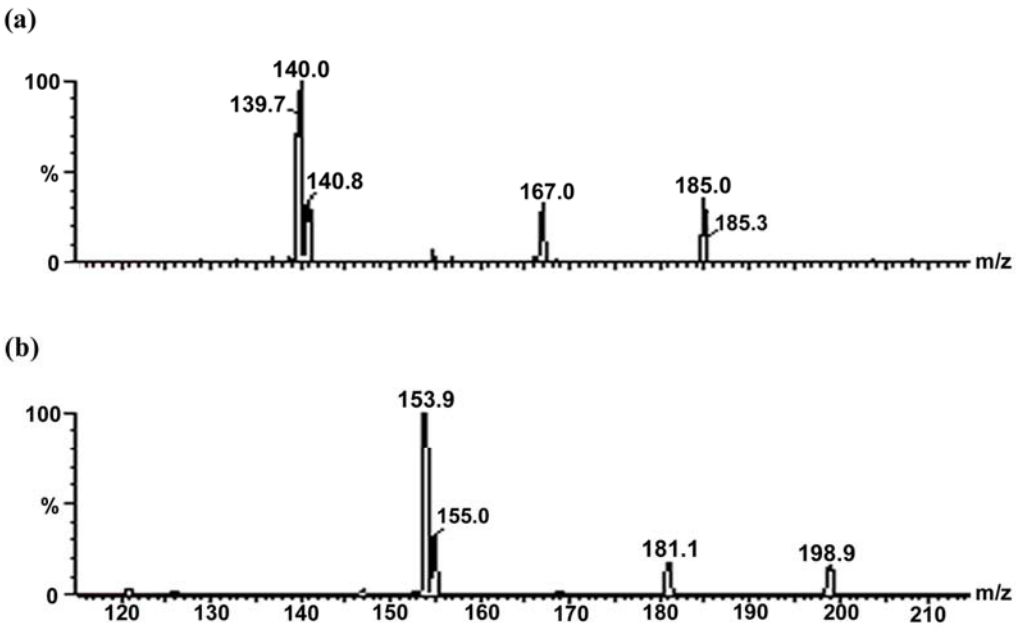

Figure 4-6. ESI-MS/MS analysis of the product of catechols catalyzed by 1,2-CTD from *C. albicans* TL3. The products of the 1,2-CTD-catalyzed reaction for catechol (a) and 4-methylcatechol (b) was disodium muconate and 3-methyl-disodium muconate, respectively.

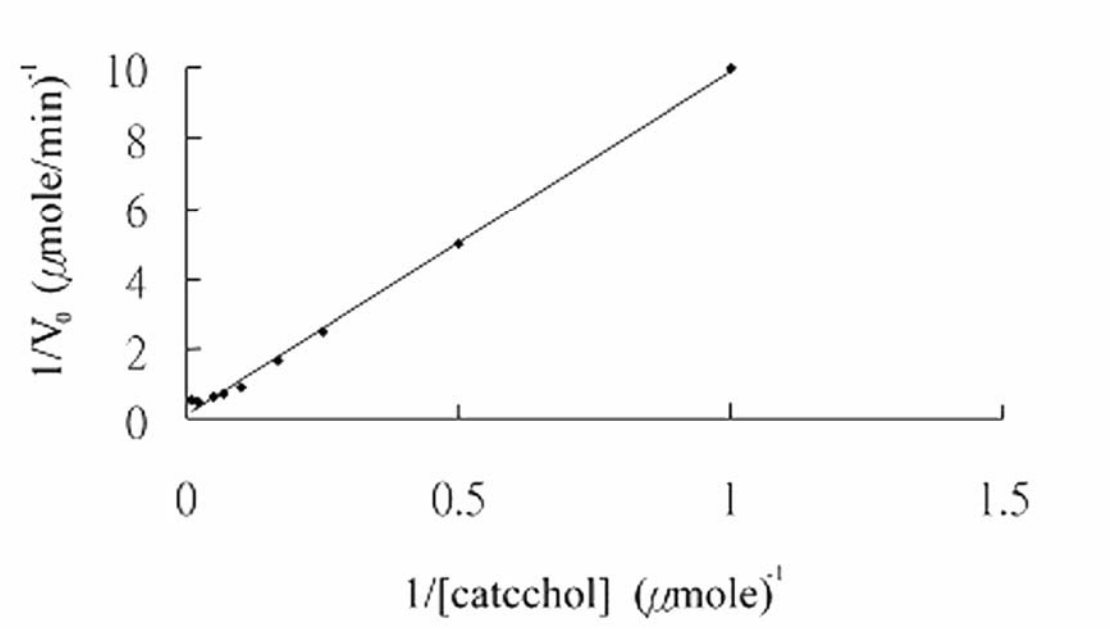

Figure 4-7. Kinetic property of 1,2-CTD from *C. albicans* TL3 for catechol.

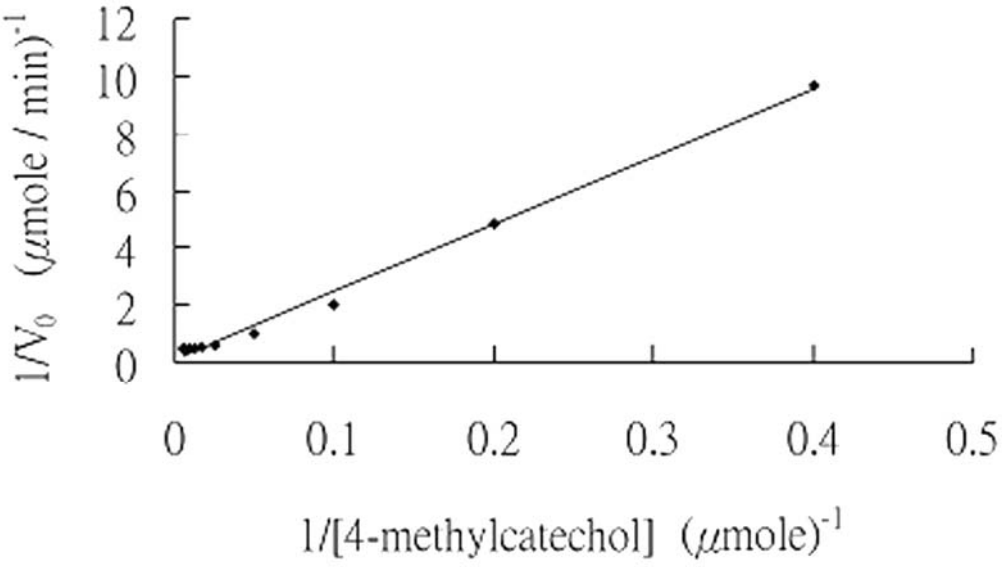

Figure 4-8. Kinetic property of 1,2-CTD from *C. albicans* TL3 for 4-methylcatechol.

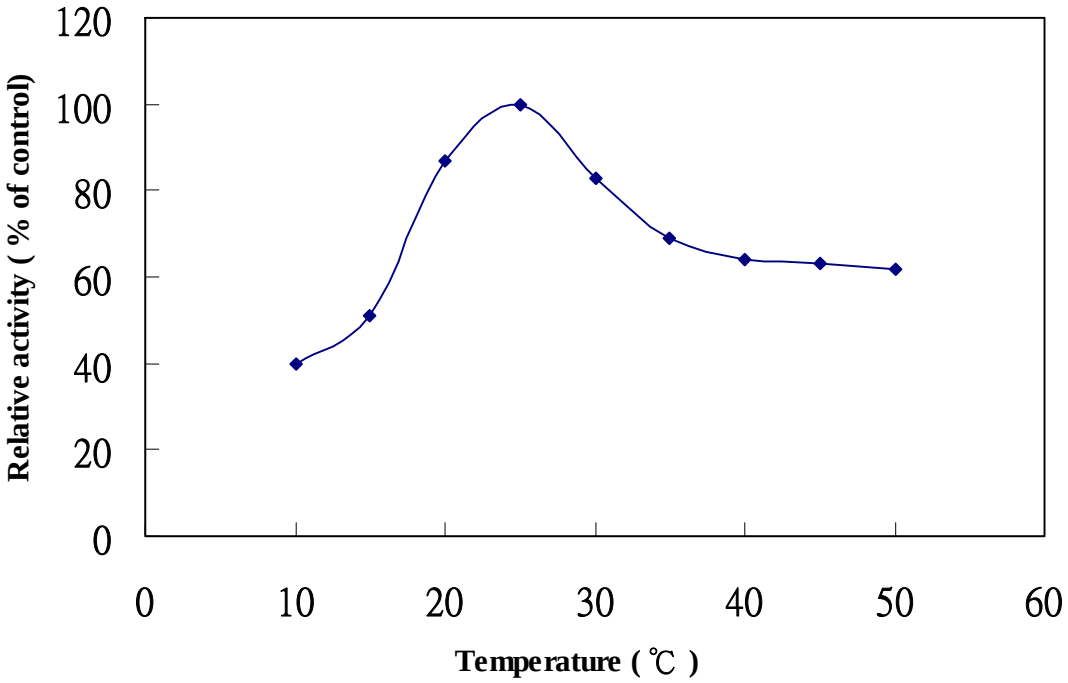

Figure 4-9. Optimal temperature of catechol 1,2-dioxygenase from *C. albicans* TL3. The relative enzyme activity was determined at designated temperature in the pH 8.3 buffer.

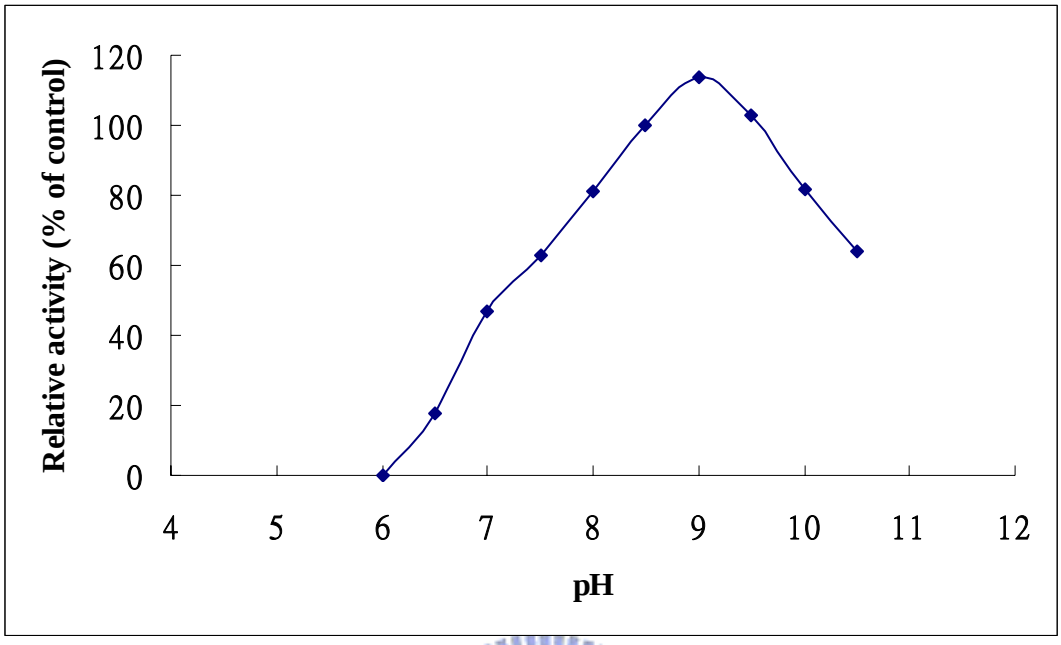

Figure 4-10. Optimal pH of catechol 1,2-dioxygenase from *C. albicans* TL3. The relative enzyme activity was determined at 25 ℃ in the designated pH of buffer.

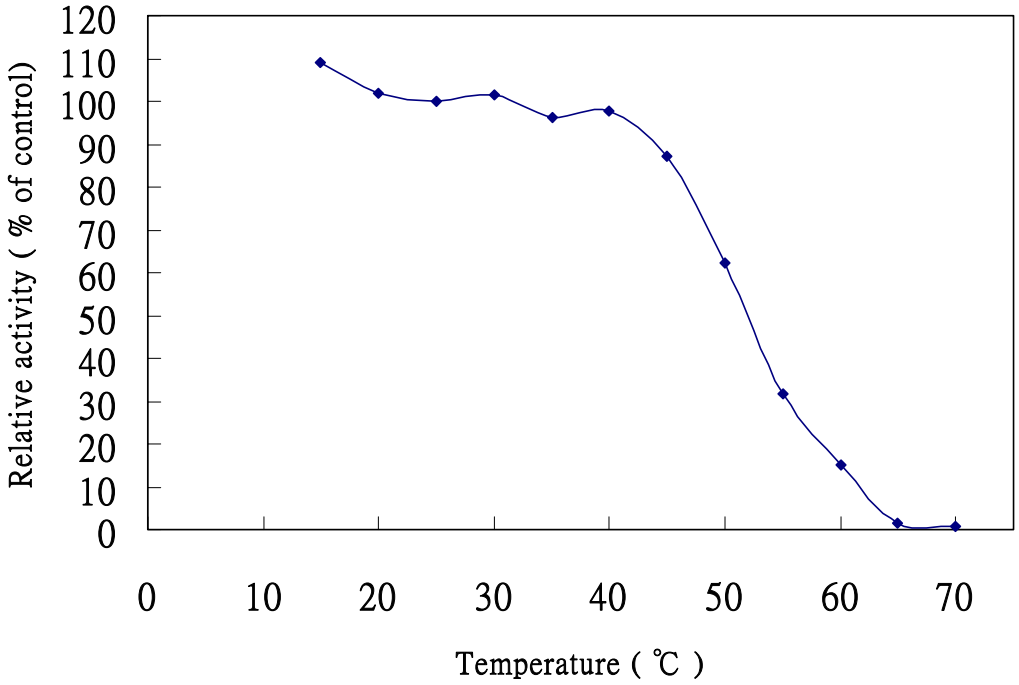

Figure 4-11. Thermal stability of catechol 1,2-dioxygenase from *C. albicans* TL3. The enzyme was incubated at indicated temperatures for 30 min at pH8.3. The remaining activity was determined by the standard assay at 25℃.

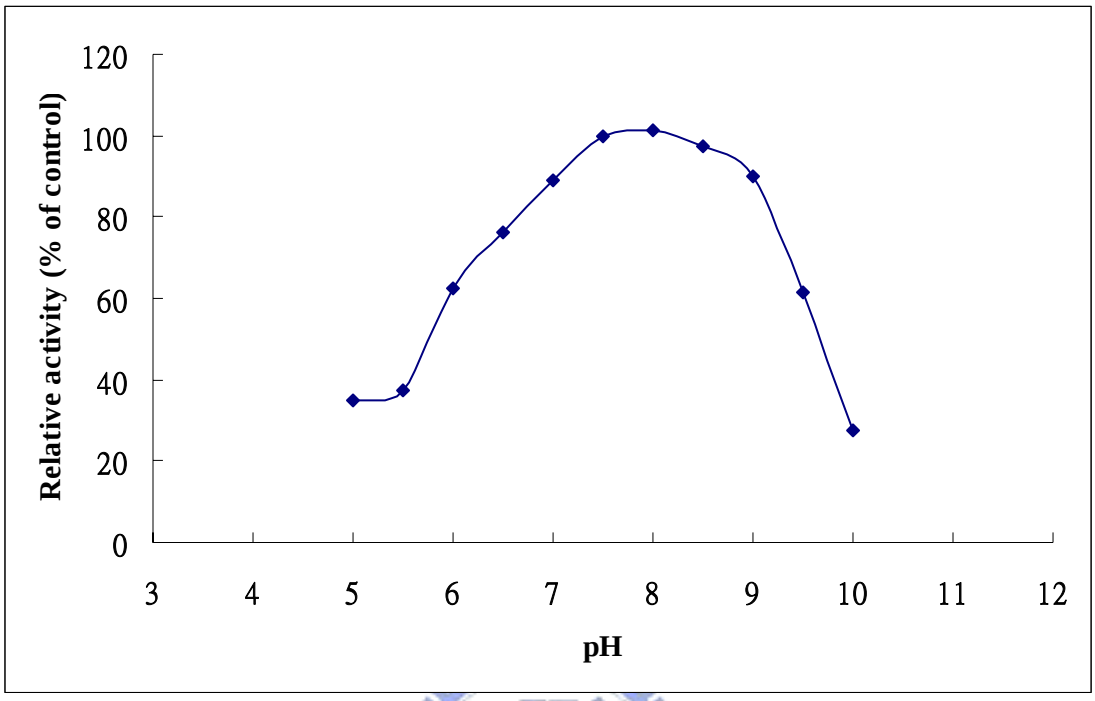

Figure 4-12. pH stability of catechol 1,2-duoxygenaase from *C. albicans* TL3. The enzyme was incubated at indicated pH for 30 min at 25℃. The remaining enzyme activity was determined by the standard assay.

Chapter 5

Proteomic analysis of a catechol 1,2-dioxygenase from a phenol degrading *Candida albicans* TL3

5.1 Abstract

2-D gel analysis of purified 1,2-CTD from *Candida albicans* TL3 revealed five spots with similar molecular weights (~32000 Da) but different pIs. These spots were subjected to further analysis using MALDI-TOF mass spectrometry. The results of MALDI-TOF analysis suggested that all of these spots were derived from the same 1,2-CTD, presumably resulting from different degrees of post-translational modification. Peptide sequencing of the fragments of 1,2-CTD by Edman degradation and MALDI-TOF/TOF analyses provided information on amino acid sequences for BLAST analysis. The BLAST analysis revealed that this eukaryotic 1,2-CTD is highly homologous (greater than 95%) to a hypothetical protein, CaO19_12036, which occurs in the same *Candida* strain and is similar to bacterial hydroxyquinol 1,2-dioxygenase.

Keywords: 1,2-CTD, *Candida albicans* TL3, 2-D gel, MALDI-TOF/TOF

5.2 Introduction

Catechols 1,2-dioxygenases play a fundamental important role in the metabolic conversion of aromatic compounds to aliphatic compounds and are ubiquitous in microorganisms (Broderick et al. 1991; Latus et al. 1995; Sauret-Ignazi et a1. 1996; Briganti et al. 1997; Strachan et al. 1998; An et al. 2001; Shen et al. 2004; Ferraroni et al. 2004; Ferraroni et al. 2005). Most catechols 1,2-dioxygenases are nonheme iron enzymes and dimer form. In generally, each subunit of catechol 1,2-dioxygenase (1,2-CTD) and hydroxyquinol 1,2-dioxygenase (1,2-HQD) is composed of the 280~310 amino acid residues, while the chlorocatechol 1,2-dioxygenase (1,2-ClCTD) is 240~260 amino acid residues. Except from the same genera microorganisms, the homology of amino acid sequence of 1,2-CTDs, 1,2-HQDs and 1,2-ClCTDs are low. However, structural studies of three family enzymes showed that the amino acid residues at the active site are highly conserved, especially those responsible for iron binding (Eulberg et al. 1997; Ridder et al. 1998; Vetting et al. 2000). Heterogeneous subunits often make 1,2-CTDs having two or three isozymes that are found in some microorganisms such as *Pseudomonas arvilla* C-1, *Acinetobacter lwoffii* K24, *Frateuria* sp. ANA-18, *Arthrobacter* sp. Ba-5-17, and *Acinetobacter radioresistens* (Aoki et al. 1984; Nakai et al. 1990; Kim et al. 1997; Murakami et al. 1998; Briganti et al. 2000).

Recently, proteomic analysis of the phenolic compounds degradation pathway and related enzymes (especially to 1,2-CTDs) in bacteria is one of popular model (Giuffrida et al. 2001; Kim SI et al. 2002, 2003). Therefore, we want to use proteomic analysis to advance the realization of 1,2-CTD, which we have purified previously from *C. albicans* TL3.

5.3 Experimental

5.3.1 2-D gel electrophoresis

The 1,2-CTD from *C. albicans* TL3 for 2-D gel electrophoresis was prepared by denaturing the purified enzyme (~30 μg) with 1 ml acetone containing 10% TCA. The precipitated protein was collected by centrifugation. The pellet was washed several times with acetone and resuspended in 300 μl sample buffer (6 M urea, 2 M thiourea, 0.5% TritonX-100, 1% DTT, and 0.5% IPG buffer). The resulting solution was applied to an immobilized pH 3–10 and pH 4–7 nonlinear gradient strip for isoelectric focusing using IPGphor (Pharmacia). The electrophoresis was carried out at five power settings: 30 V for 12 h, 100 V for 2 h, 500 V for 2 h, 1000 V for 2 h, and 8000 V for 8 h. The second dimension was performed using 12.5% SDS-PAGE (Hoefer SE 600, Amersham Biosciences, USA). The gel was stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich).

5.3.2 In-gel digestion

The stained protein spots excised from the 2-D PAGE gel were dehydrated three times using 50% acetonitrile and 0.2 M ammonium bicarbonate and then digested with trypsin (Promega) in 0.2 M ammonium bicarbonate containing 0.02% Tween-20 at 37 °C for 16 h.

5.3.3 N-terminal protein sequencing

The five major protein spots separated on the 2-D gel were transferred onto a PVDF membrane (Problott, Applied Biosystems, USA) at 400 mA for 1 h using a TE2 transphor electrophoresis unit (Hoefer, Amersham Biosciences, USA) with 10 mM CHAPS buffer (pH 11) containing 10% methanol. The membrane was stained with Coomassie Brilliant Blue R250 solution and washed with 50% methanol. Stained protein spots were excised from the membrane and installed in the blot cartridge of a Procise 494 sequencer (Applied Biosystems, USA) for amino acid sequencing.

5.3.4 Peptide sequencing by MALDI-TOF

Trypsinized peptides were dissolved in 0.5% TFA solution and then eluted onto the MALDI target plate using 50% acetonitrile containing 0.5% TFA and 10 mg/ml α-cyano-4-hydroxycinnamic acid. Sample peptides were analyzed by TOF-MS and TOF-MS/MS (4700 proteomics analyzer, Applied Biosystems, Framingham); UV light (355 nm) was provided by an Nd:YAG laser with a 200 Hz repetition rate. For peptide sequencing by MS/MS analysis, collision-induced dissociation was performed using air as the collision gas. The collision energy was set to 1 kV and mass spectra were analyzed using the Mascot program and the NCBI nonreductant database. MS/MS spectra were analyzed using DeNovo Explores (TM) software (Version 3.5).

5.4 Results and Discussion

5.4.1 *MALDI-TOF* analysis of the isotypes of 1,2-CTD

A 2-D gel electrophoresis was employed for analyzing the purity of 1,2-CTD. Five major protein spots with nearly identical molecular weights (~32,000 Da) but slightly different pI values (5.3−5.7) were observed in the acidic region of the 2-D gel (Fig. 5-1, 5-2). The pI value of this enzyme was slightly higher compared to previous pI values (4.2−5.2) of 1,2-CTDs from prokaryotic cell (Aoki et al. 1984; Sauret-Ignazi et a1. 1996; Briganti et al. 1997; Giuffrida et al. 2001; Kim et al. 2003). These protein spots were digested in-gel with trypsin before MALDI-TOF analyses were performed. The peptide fragmentation patterns derived from these protein spots were almost identical (Fig. 5-3), indicating that the five protein spots (isotypes) were probably derived from the same gene. The reason for the slight differences in pI between isotypes of 1,2-CTD is unknown. Although it is likely that the isotypes of 1,2-CTD resulted from post-translational modifications, further study is required to elucidate this enigma. Similarly, isotypes of 1,2-CTD were found in *Acinetobacter lowffii* K24 (Kim et al. 2002).

5.4.2 Amino acid sequence analysis of 1,2-CTD

To identify the amino acid sequence of 1,2-CTD from *C. albicans* TL3, the five

isotypes that separated on the 2-D gel were electroblotted onto a PVDF membrane and then subjected to N-terminal sequencing by automatic Edman degradation. Unfortunately, all five isotypes seemed to be N-terminal blocked, a feature that is not exhibited by most 1,2-CTDs of bacteria (Sauret-Ignazi et al. 1996; Briganti et al. 1997; Pessione et al. 2001; Kim et al. 2003). Recently, a database of 1,2-CTDs from many microorganisms, including *Acinetobacter*, *Bradyrhizobium*, *Corynebacterium*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*, has been established. However, the Blast search of MALDI-TOF mass spectrometry showed that 1,2-CTD from *C. albicans* TL3 does not have any significant similarity to proteins currently available in the NCBI database. Therefore, we suggest that the primary structure of 1,2-CTD from *C. albicans* TL3 may be very different from those of bacteria.

The trypsin-digested peptides from purified 1,2-CTD were further isolated by RP-HPLC separation (Fig. 5-4) and sequenced by an automatic Edman sequencer. Two peptides (fragments 1 and 2 in Fig. 5-7) were unequivocally sequenced. Based on these sequences, a Blast analysis was performed. A hypothetical protein — CaO19_12036 from *Candida albicans* SC5314 (GenBank accession no. XM 717691) was hit with a very low E value (1e-09). In addition, MALDI-TOF/TOF mass spectrometry provided high-scoring (above 80) de novo sequences of two fragments with m/z of 932 Da (fragment 4) and 1199 Da (fragment 3) (Fig. 5-5, 5-6).

These sequences were identical to that of the hypothetical protein, which is suggested to be similar to the bacterial hydroxyquinol 1,2-dioxygenase. Thus, we believe that this hypothetical protein of *C. albicans* SC5314 is indeed a 1,2-CTD. Recently, Earhart et al. have determined the residues form the active sites of 1,2-CTD, 1,2-ClCTD and 1,2-HQD family from prokaryotic cell (Ferraroni et al. 2004; Ferraroni et al. 2005; Earhart et al. 2005). A multiple sequence alignment of various 1,2-CTDs revealed 1,2-CTD from *C. albicans* SC5314 had high conserved residues in the active site, especially in iron-binding site residues are absolutely conservation (Fig. 5-8). This result perhaps will be helpful to explain why 1,2-CTDs from *C. albicans* TL3. and bacteria are significantly different in sequence but with the same catalytic function. Interestingly, 1,2-CTD of *C. albicans* was more close to 1,2-ClCTD of *R. opacus* 1CP (only one residue difference : Val 254/Cys 224) than other bacterial 1,2-CTDs in residues within the active site. It is likely that this position residue (Val 254/Cys 224) maybe responsible for the substrate selectivity difference between1,2-CTD and 1,2-ClCTD.

5.5 Conclusion

On the basis of MALDI-TOF mass spectrometry analysis, we suggest that the five 1,2-CTD isotypes from *C. albicans* TL3 that were separated on 2-D gel represent various levels of post-translational modification of the same enzyme. We confirmed that hypothetical protein CaO19_12036 from *C. albicans* SC5314 is indeed 1,2-CTD, which is very different, in terms of primary structure, from those of bacterial enzymes. As compared with the 1,2-ClCTD from *R. opacus* 1CP, the Val 254 of 1,2-CTD from *C. albicans* may be critical for substrate recognition. This study provides useful genetic information, which can facilitate the gene cloning of 1,2-CTD from *Candida albican*s TL3.

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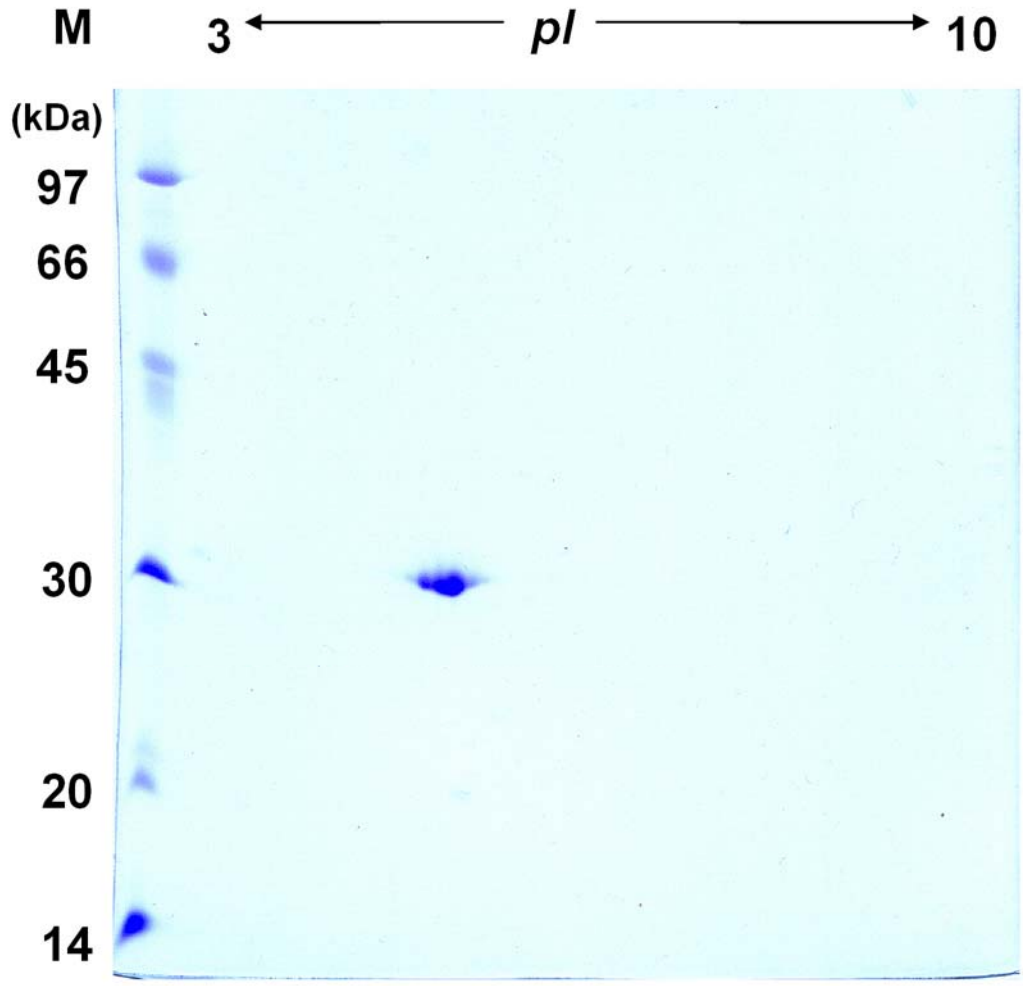

Figure 5-1. 2-D gel electrophoresis of 1,2-CTD from *C. albicans* TL3. The protein were separated on the 2-D gel by electrofocusing using an IPG strip (pH 3–10 NL) and 12.5% SDS-PAGE.

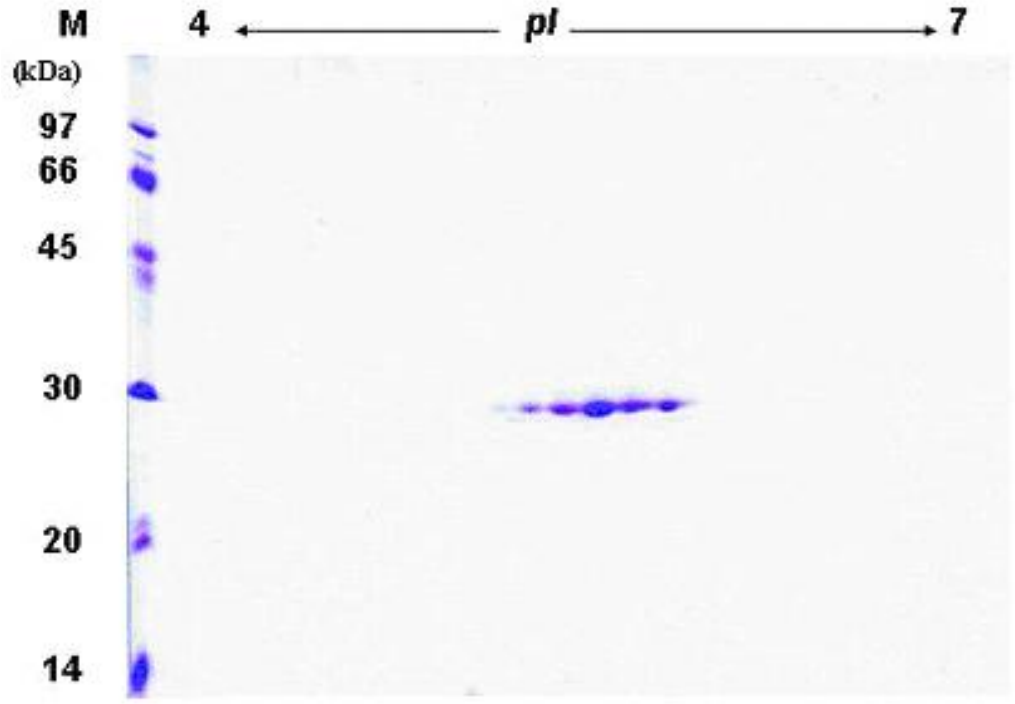

Figure 5-2. 2-D gel electrophoresis of 1,2-CTD from *C. albicans* TL3. The protein were separated on the 2-D gel by electrofocusing using an IPG strip (pH 4 –7 NL) and 12.5% SDS-PAGE.

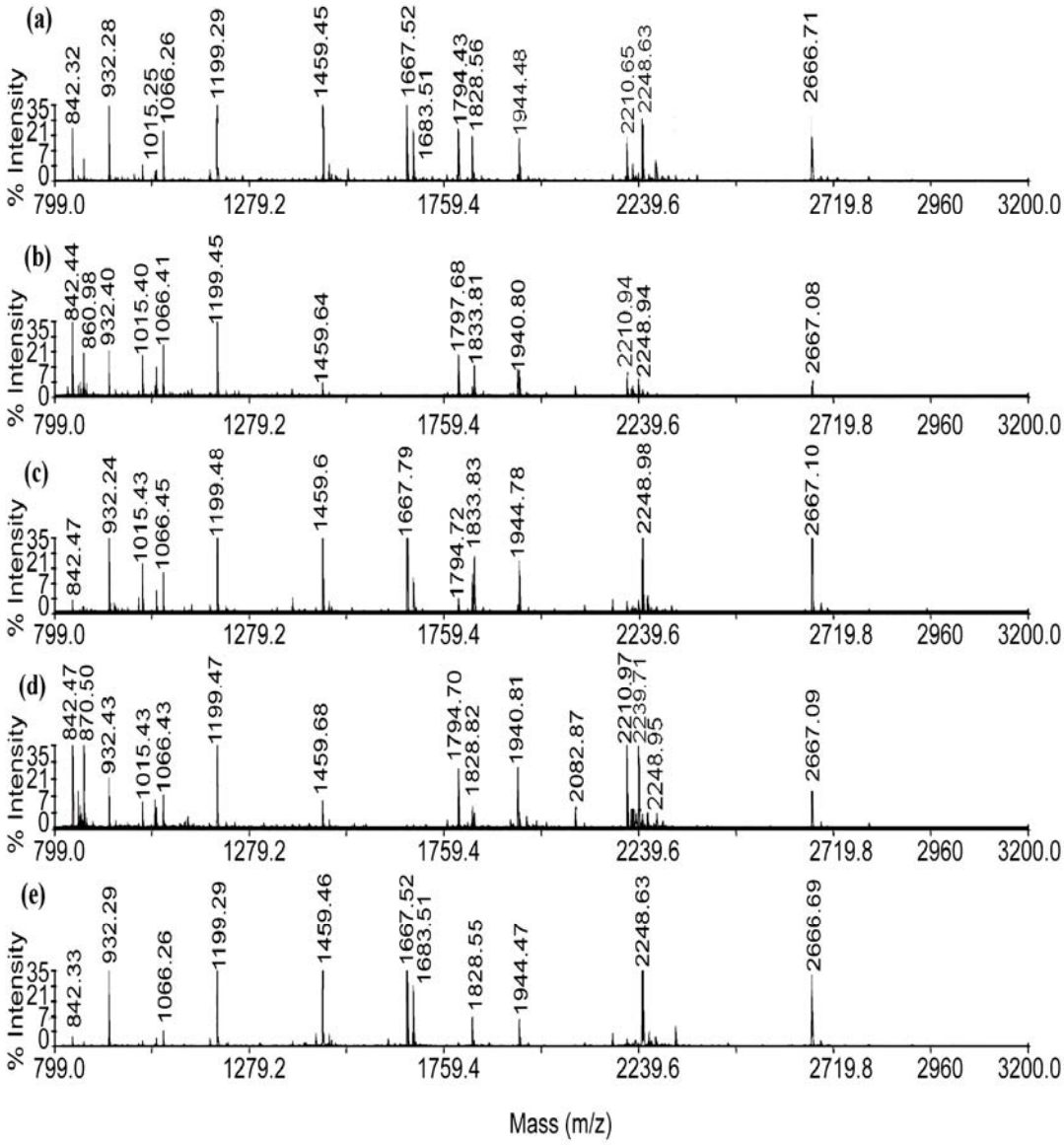

Figure 5-3. MALDI-TOF mass spectrometry analysis of five 1,2-CTD isotypes from *C. albicans* TL3 on the 2-D gel. (a)~(e) represent isotypes from left to right on the 2-D gel, respectively.

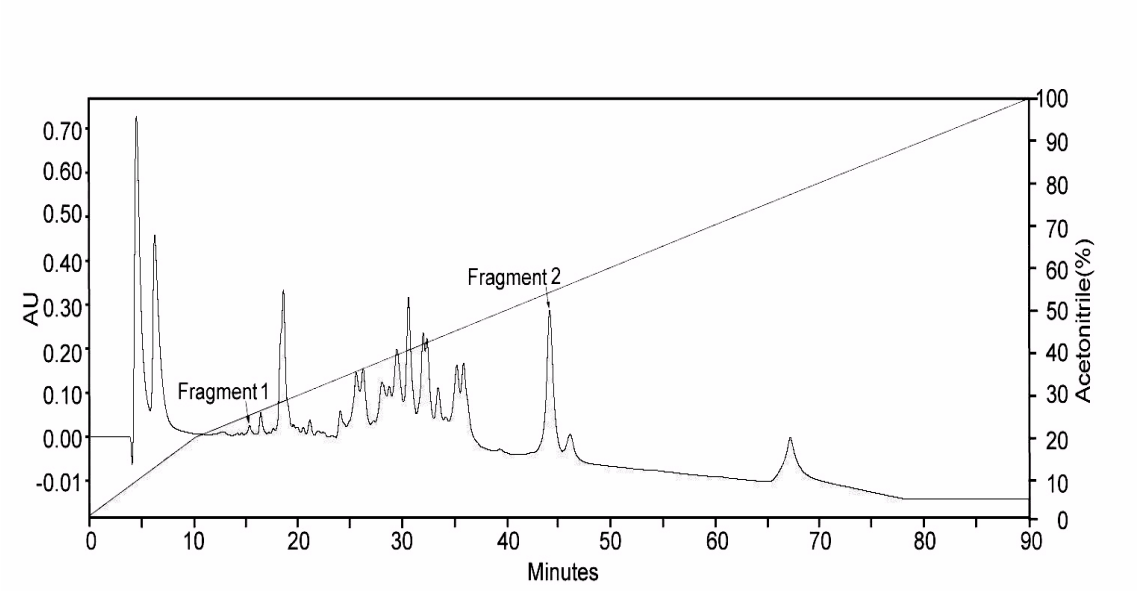

Figure 5-4. RP-HPLC separation of fragments from trypsin-digested 1,2-CTD of *C. albicans* TL3. Peaks of fragment 1 and fragment 2 were manually collected at 210 nm and used for sequencing by Edman degradation.

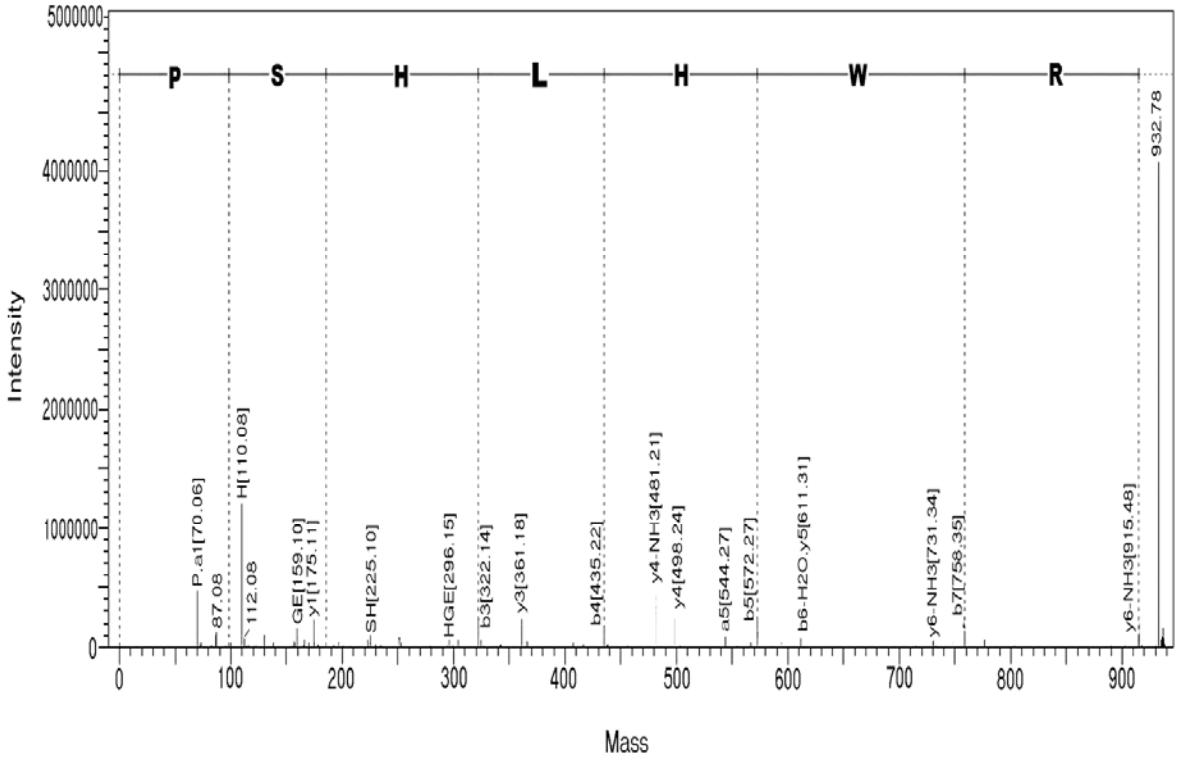

Figure 5-5. De novo sequences of peptide fragment with m/z 932 Da derived from 1,2-CTD from *C. albicans* TL3. The fragment was sequenced by MALDI-TOF/TOF mass spectrometry analysis.

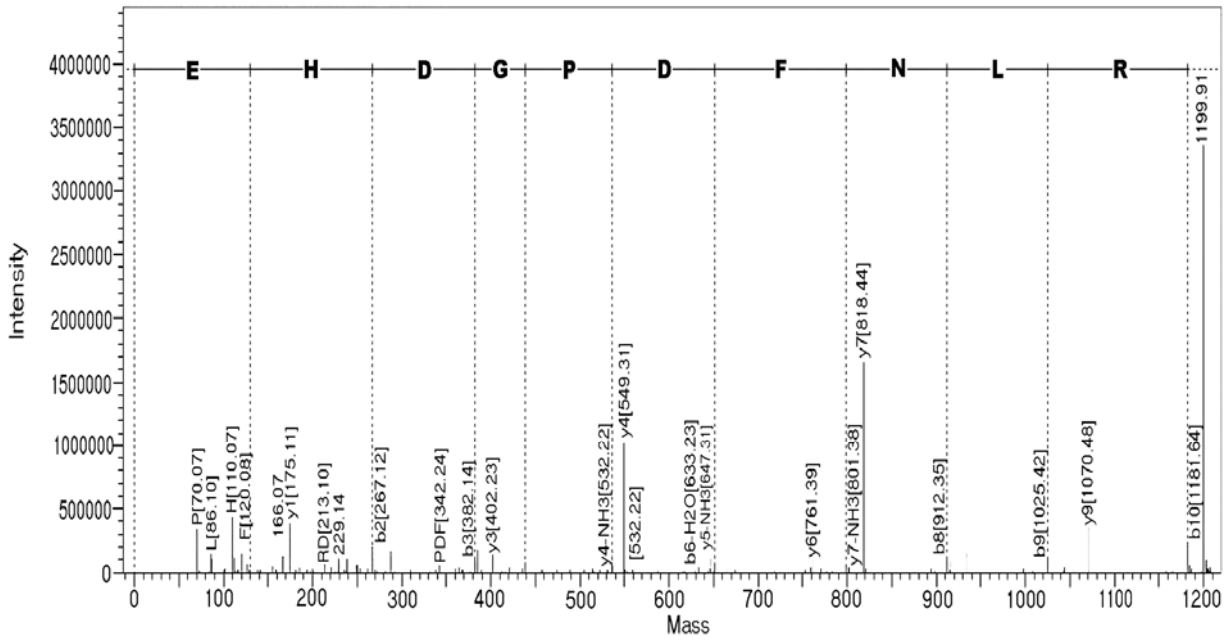

Figure 5-6. De novo sequences of peptide fragment with m/z 1199 Da derived from 1,2-CTD from *C. albicans* TL3. The fragment was sequenced by MALDI-TOF/TOF mass spectrometry analysis.

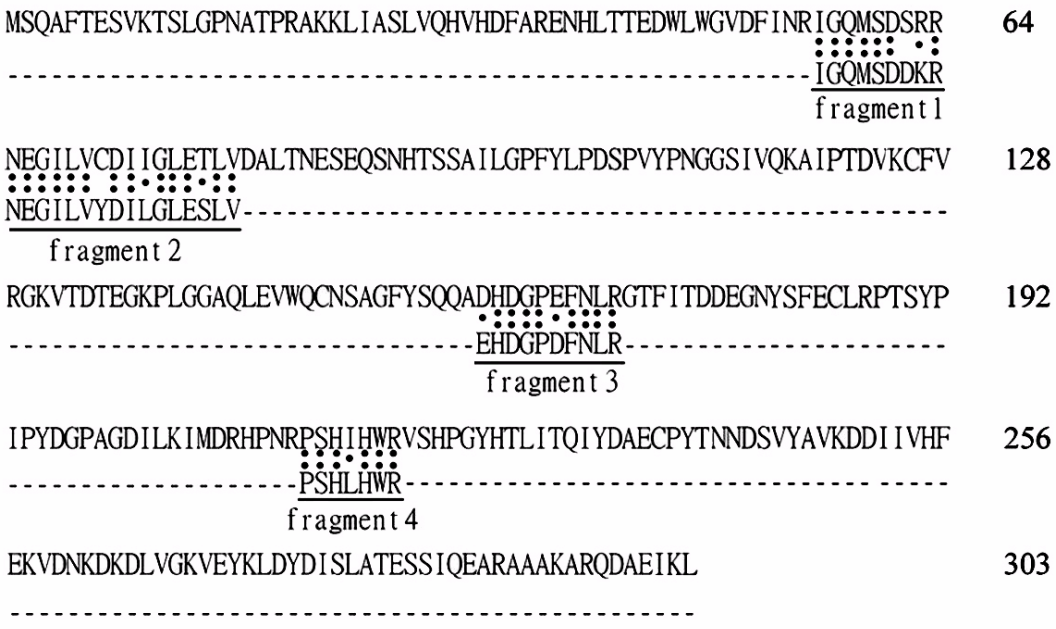

Figure 5-7. Internal amino acid sequence homology of 1,2-CTD of *C. albicans* TL3 with hypothetical protein CaO19_12036 of *C. albicans* SC5314 (XP_722784 XP_431250). Amino acid sequence of fragment 1 and fragment 2 were determined by Edman sequencer. Amino acid sequence of fragment 3 and fragment 4 were candidated by MALDI-TOF/TOF mass spectrometry. These fragments all are trypsinized peptides from *C. albicans* TL3.

Figure 5-8. Amino acid sequence alignment of 1,2-CTDs and 1,2-ClCTD. 1, *Acinetobacter calcoaceticus* ADP-1 1,2-CTD (NCBI protein accession P07773); 2, *Pseudomonas arvilla* C-1(α subunit)1,2-CTD (NCBI protein

accession AAN68774); 3, *Bradyrhizobium* sp. BTAi1 1,2-CTD (NCBI protein accession ZP_00861819); 4, *Streptomyces setonii* 1,2-CTD (NCBI protein accession AAN76673); 5, *Corynebacterium glutamicum* ATCC 13032 1,2-CTD (NCBI protein accession YP_227304); 6, *Candida albicans* SC5314 1,2-CTD (hypothetical protein CaO19_12036) (NCBI protein accession XP_722784 XP_431250); 7, *Rhodococcus opacus* 1CP1,2-ClCTD (NCBI protein accession CAD28142). Shaded residues form the active site. The strongly conserved positions are indicated in the following ways: asterisk (*), positions that have a single, fully conserved residue; colon (:), positions with fully conserved strongly groups; dot (.), positions with fully conserved weaker; (#), positions are the iron-binding sites.