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

San-Chin Tsai · Yaw-Kuen Li

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Abstract A eukaryotic catechol 1,2-dioxygenase (1,2-CTD) was produced from a *Candida albicans* TL3 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. The optimal temperature and pH were 25°C and 8.0, respectively. Substrate analysis showed that the purified enzyme was a type I catechol 1,2-dioxygenase. This is the first time that a 1,2-CTD from a eukaryote (*Candida albicans*) has been characterized. Peptide sequencing on fragments of 1,2-CTD by Edman degradation and MALDI-TOF/TOF mass analyses provided information of amino acid sequences for BLAST analysis, 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. We conclude that the hypothetical protein is 1,2-CTD.

Keywords Catechol 1,2-dioxygenase · *Candida albicans* TL3 · MALDI-TOF mass analysis

S.-C. Tsai · Y.-K. Li (✉)
Department of Applied Chemistry,
National Chiao Tung University, 1001 Ta-Hseh Rd.,
Hsinchu, Taiwan
e-mail: ykl@cc.nctu.edu.tw

S.-C. Tsai
Department of Medical Technology,
Yuanpei University, Hsinchu, Taiwan

Introduction

Catechols are formed during biodegradation of a variety of aromatic compounds by aerobic microorganisms. The aromatic rings of catechols may be cleaved by intradiol dioxygenase via an ortho-cleavage pathway to form cis,cis-muconate or by extradiol dioxygenase via a meta-cleavage pathway to form 2-hydroxymuconic semialdehyde. These metabolites are degraded in the tricarboxylic acid cycle (Ngai et al. 1990; Chen and Lovell 1990; Aoki et al. 1990). Both types of catechol dioxygenase use nonheme iron as a cofactor (Nozaki 1979). Intradiol dioxygenases are classed into two structurally different families: catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases (3,4-PCDs), which are specific for catechol (or its derivatives) and hydroxybenzoates, respectively. In general, catechol 1,2-dioxygenases are dimeric proteins with identical or similar subunits. Catechol 1,2-dioxygenases are classified into two types according to their substrate specificities: type I enzymes are specific for catechols, alkylated catechols (catechol 1,2-dioxygenase, 1,2-CTD) (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-dioxygenase, 1,2-HQD) (Latus et al. 1995); type II enzymes are specific for chlorocatechols (chlorocatechol 1,2-dioxygenase, 1,2-ClCTD) (Broderick et al. 1991; Van der Meer et al. 1993; Maltseva et al. 1994).

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, 2005). 1,

2-CTDs from bacteria have been extensively characterized in terms of their biochemical and structural properties and their amino acid sequences (Kim et al. 1997, 2002, 2003;Eulberg et al. 1997; Feng et al. 1999; Pessione et al. 2001; Caposio et al. 2002; Kim et al.; Earhart et al. 2005). Various 1,2-CTD 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). However, structural studies of 1,2-CTDs 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).

Although there are many reports concerning 1,2-CTDs, they are almost all about enzymes from prokaryotes. Previously, we isolated a strain of yeast, *Candida albicans*, 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). In the present study, we extended our previous findings by purifying and characterizing 1,2-CTD that may cleave the catechol ring derived from the phenol degradation.

Materials and methods

Chemicals

Chemicals were purchased from Sigma (St Louis, MO) or Merck (Darmstadt, Germany). Yeast nitrogen base (YNB) without amino acids was obtained from Difco (Detroit, MI). All other chemicals used were reagent grade. Buffers used for various pH conditions were sodium acetate, pH 5.0–6.0; potassium phosphate, pH 6.5–8.5; Tris–acetate, pH 9.0–10.0.

Purification of catechol 1,2-dioxygenase

Cultures were grown in eight 2-L Erlenmeyer flasks at 30°C. Each flask, containing 800 ml of YNB medium (0.67% w/v) and 10 mM phenol (Tsai et al. 2005), was inoculated with an initial OD_{600} of 0.01 of *C. albicans* TL3. Cells were harvested when the growth of the strain approached the stationary phase (OD_{600} of approximately 1.4). The harvested cells were washed twice with distilled water and resuspended in 34 ml of buffer A (50 mM Tris–HCl, pH 8.3, containing 5 mM β-mercaptoethanol) and then disrupted by sonication. After centrifugation to remove cell debris, the supernatant was subjected to protein precipitation. The precipitant obtained from 50 to 70% saturation of ammonium sulfate was collected and resuspended in buffer A (3 ml) for gel filtration chromatography (Sephadex G-75, 2 × 80 cm). The column was eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions, eluted from 610 to 680 min, with 1,2-CTD activity were pooled and concentrated by centrifugal ultrafiltration (Amicon MWCO 10 kDa, Millipore). All the concentrated sample (5 ml) was further loaded onto the HiTrap Q Sepharose column (5 ml × 2; Amersham Bioscience, Uppsala, Sweden), pre-equilibrated with buffer A. Chromatography was performed with a linear gradient from 0 to 0.2 M of $(NH_4)_2SO_4$ in buffer A at a flow rate of 0.5 ml/min. The active fractions, eluted at 0.15–0.16 M of $(NH_4)_2SO_4$, were analyzed on enzyme purity by SDS-PAGE and pooled for further study. All purification steps were carried out at 4°C.

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.

Determination of molecular weight

The molecular weight of the subunit of 1,2-CTD was determined using 12.5% SDS-PAGE as described by Laemmli (Laemmli 1970). 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) 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 2,500 units with a scan step of 2 s and an interscan of 0.1 s 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 5–10 μg of protein in desalted form were used for MS analysis.

Enzyme activity assays

1,2-CTD activity was determined by measuring the increase of absorbance at 260 nm, corresponding to the

formation of products from all tested substrates except for hydroxyquinol and protocatechuate, for which wavelengths of 245 nm and 270 nm were employed, respectively (Varga and Neujahr 1970; Bull and Ballou 1981; Strachan et al. 1998). One unit of 1,2-CTD was defined as the amount of enzyme needed to produce 1 μmol of product per minute. The standard assay of enzyme activity was performed by addition of 2 μl (68 μg/ml) of 1,2-CTD to 998 μl of assay solution (buffer A containing 20 μM $FeSO_4$ and 0.1 mM of catechol) at 25°C. Kinetic parameters, k_{cat} and K_m, were estimated from the double reciprocal plots of specific rate versus substrate concentration. To investigate the thermal and pH stabilities of the enzyme and the effects of various metal ions on 1,2-CTD activities, the enzyme was pre-incubated at various temperatures, pH values, or metal ions for 30 min. The residual enzyme activity was subsequently measured with standard assay.

Iron analysis

This 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.

Determination of amino acid sequence

For amino sequence analysis, the purified 1,2-CTD was digested by trypsin (Promega Co.) in the condition of 0.2 M of ammonium bicarbonate containing 0.02% Tween-20 at 37°C for 16 h. The trypsinized peptides were separated by reverse phase HPLC using a reverse-phase column (Waters, *u* Bondapak C_{18}, 3.9 × 300 mm) with two linear gradients of acetonitrile containing 0.1% TFA (0–20% and 20–100% in 10 and 80 min, respectively). Each peak was manually collected and used for Edman sequencing (Procise 494 sequencer, Applied Biosystems, USA).

Trypsinized peptides were also 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, USA). For peptide sequencing by MS/MS analysis, collision-induced dissociation was performed using air as the collision gas and the collision energy was set to 1 kV. The resulted mass spectra were analyzed by using the Mascot program to search in NCBI nonreductant database and by using DeNovo Explores (TM) software (Version 3.5) to predict the amino acid sequence.

Results and discussion

Purification of 1,2-CTD

In our previous study, we have demonstrated that the presence of phenol in cultural medium is essential for the induction of 1,2-CTD activity. The optimal induction was found when *C. albicans* TL3 was cultivated in a medium containing 10 mM phenol (Tsai et al. 2005). In this study, similar condition, shown in the experimental section, was employed to produce 1,2-CTD. Purification of 1,2-CTD from cell-free extracts was performed using ammonium sulfate precipitation and Sephadex G-75 gel filtration followed by HiTrap Q Sepharose column chromatography as described inMaterials and methods. Yields for each step of the purification process are summarized in Table 1. Forty-fold purification of this 1,2-CTD was achieved with a yield of 33%. The specific activity of the purified enzyme was 63.0 units per mg protein. Gel-filtration analysis using a Sephadex G-75 column showed that the purified enzyme eluted at a position corresponding to a molecular weight of about 64 kDa. After SDS-PAGE, the purified enzyme appeared as a single band with a molecular weight of 31 kDa (Fig. 1), suggesting that the enzyme is a dimeric protein. The molecular weight of the subunit of 1,2-CTD was measured by ESI-MS to be 31,994 ± 2 Da (Fig. 2), 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 bacteria, which have molecular weights of 30,500–34,000 Da per subunit (Eck and Bettler 1991; Neidle et al. 1988).

Characterization of 1,2-CTD

The 1,2-CTD of *C. albicans* TL3 showed considerable activity towards catechol and 4-methylcatechol (18% of that of catechol), but no significant activity towards other catechol derivatives such as 3-methycatechol, 4-chlorocatechol, 4-carboxycatechol, and hydroxyquinol. The products of the enzymatic reaction were analyzed and confirmed by negative mode of ESI-MS and tandem MS (data not shown). The catalytic product of 1,2-CTD towards catechol (or 4-methylcatechol) showed a signal at *m/z* 185 (or 199), which is consistent with the molecular weight of disodium muconate (or disodium 3-methylmuconate). The tandem mass analysis of *m/z* 185 showed the fragments with *m/z* 140 and 167, which were found to be completely identical to the fragment pattern of pure *cis, cis*-disodium muconate. Similarly, the MS/MS fragments derived from the peak of *m/z* 199 were 154 and 181, which is

Table 1 Purification of 1,2-CTD from *Candida 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

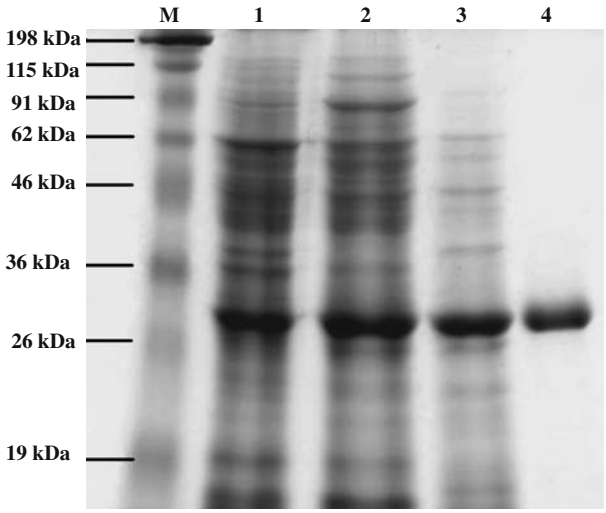

Fig. 1 SDS-PAGE analysis of 1,2-CTD from *C. albicans* TL3 in various steps of purification. *Lane M*, protein markers; *Lane 1*, crude cell extract, 170 μg protein; *Lane 2*, 50–70% $(NH_4)_2SO_4$ precipitation, 26 μg protein; *Lane 3*, after chromatography on a G-75 column, 4 μg protein; *Lane 4*,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

14 *amu* larger than those from disodium muconate. Thus, we concluded that the catalytic end product derived from 4-methylcatechol (*m/z* 199) is disodium 3-methylmuconate. 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.

The optimal temperature and pH of 1,2-CTD from *C. albicans* TL3 was 25°C and pH 8.0, respectively. 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 purified enzyme was stable (maintaining > 85% activity) for at least 30 min at the pH range of 7.0–9.0, whereas the stability of enzyme greatly decreased in the pH

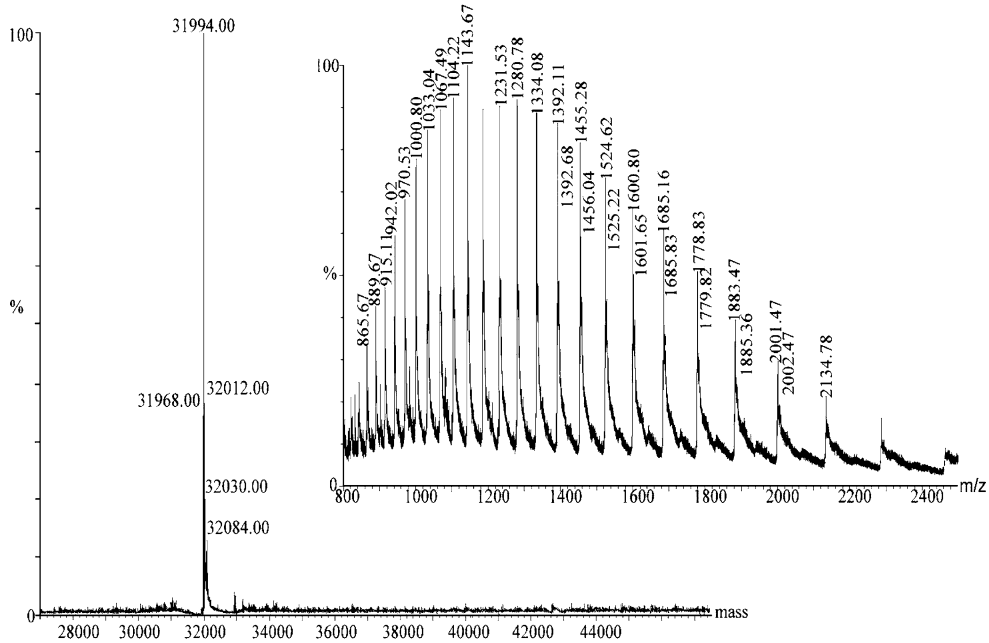

Fig. 2 The mass spectrum of the purified 1,2-CTD from *C. albicans* TL3 (*inset*) and the deconvolution of the spectrum to give a molar mass of 31,994 amu

condition out of this range (Fig. 3). The enzyme was found to be stable when it was kept at temperature lower than 40°C (Fig. 4). The biochemical properties of 1,2-CTD from *C. albicans* TL3 are summarized in Table 2.

The effects of metal salts and chelating agents on the activity of this enzyme were investigated. The results are shown in Table 3. The activity of this enzyme towards 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%. The strong inhibition (>90%) of $AgNO_3$ towards 1,2-CTD activity was also commonly observed in bacteria, such as that from *Frateuria* species ANA-18 (Aoki et al. 1984) and *Acinetobacter* sp. KS-1 (Kim et al. 2003).

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) per 0.0127 (±0.0005) nmol (determined by Bradford method) or 0.0133 (±0.0007) nmol (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.

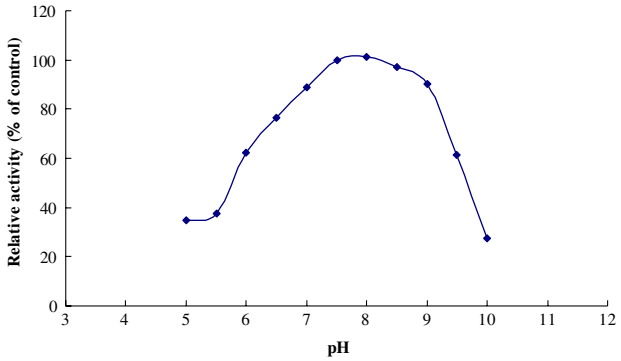

Fig. 3 The pH stability of 1,2-CTD from *C. albicans* TL3. The enzyme was incubated at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 for 30 min at 25°C. The residual activity of enzyme was determined by the standard assay and compared with the activity of the enzyme kept at 25°C, pH 8.3

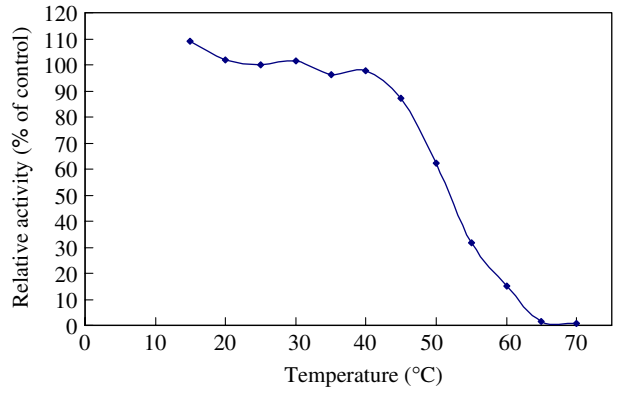

Fig. 4 Thermal stability of 1,2-CTD from *C. albicans* TL3. The enzyme was incubated at 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70°C for 30 min at pH 8.3. The residual activity of enzyme was determined by the standard assay and compared with the activity of the enzyme kept at 25°C, pH 8.3

Table 2 The properties of catechol 1,2-dioxygenase from *Candida albicans* TL3

Molecular weight (Da)	
SDS-PAGE	31,000
ESI-MS	31,994
Gel filtration	64,000
Iron content (mol/mol enzyme)	2
Optimal pH	8.0
Optimal temperature	25°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 3 Effects of some metal ions and compounds on catechol 1,2-dioxygenase activity from *Candida 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

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

Amino acid sequence analysis

Since the eukaryotic 1,2-CTD has not yet been extensively studied, N-terminal sequence by Edman degradation was performed. Unfortunately, the purified enzyme seemed to be N-terminal blocked, a feature

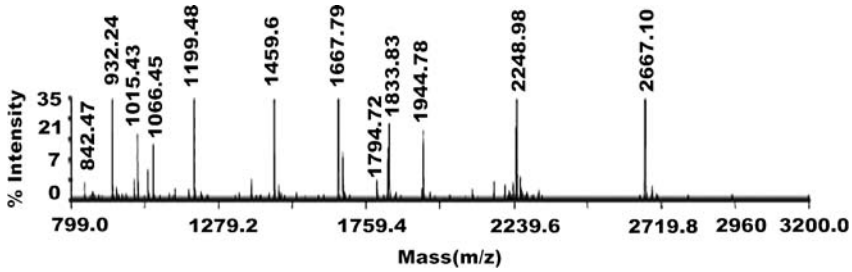

Fig. 5 MALDI-TOF mass spectrometry analysis of the 1,2-CTD from *C. albicans* TL3

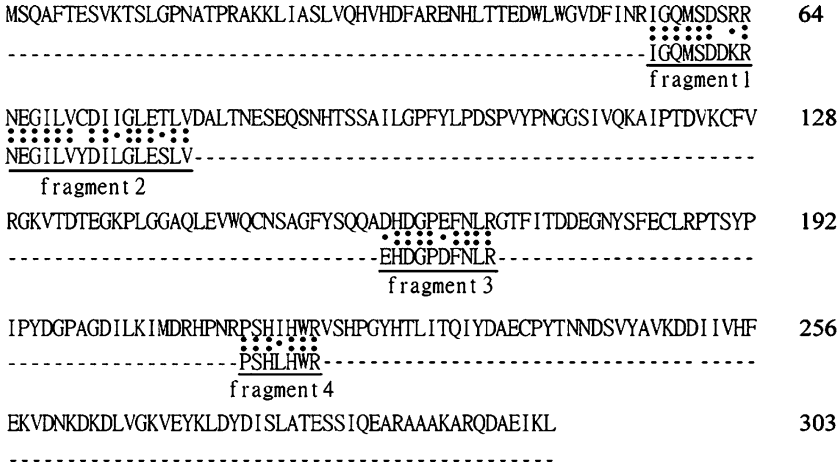

Fig. 6 Internal amino acid sequence homology of 1,2-CTD of *C. albicans* TL3 with hypothetical protein CaO19_12036 of *C. albicans* SC5314 (GenBank accession no. XM 717691). Amino acid sequences of *fragments* 1 and 2 were determined by an Edman sequencer. Amino acid sequences of *fragments* 3 and 4 were determined by MALDI-TOF/TOF mass spectrometry. The fragments were trypsinized peptides from *C. albicans* TL3

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). A Blast, based on the MALDI-TOF mass spectrometric analysis of the trypsin-digested 1,2-CTD (Fig. 5), was then performed but failed to find any available protein in the NCBI database with significant similarity to 1,2-CTD. We further isolated the trypsin-digested peptides from purified 1,2-CTD by RP-HPLC separation. Two peptides, eluted at 15 and 44 min (designated as fragments 1 and 2, respectively, in Fig. 6), were collected and unequivocally sequenced by an automatic Edman sequencer. The sequences of fragment 1 and 2 were highly matched to a hypothetical protein, CaO19_12036, from *Candida albicans* SC5314 (GenBank accession no. XM 717691) (Fig. 6). In addition, MALDI-TOF/TOF mass spectrometry (data not shown) provided high-scoring (above 80) de novo sequences of two fragments (fragments 3 and 4 in Fig. 6) with *m/z* of 932 Da and 1,199 Da in Fig. 5 and these sequences also appeared to be highly identical with this hypothetical protein. Therefore, we suggested this hypothetical protein CaO19_12036 of *C. albicans* SC5314 (XP_722784 XP_431250) should be 1,2-CTD. It is possible that may have other gene(s) in *C. albicans* encoding 1,2-CTD(s). However, since the complete genome of this strain has not yet available, this argument cannot be evaluated at the current stage.

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, and was similar to bacterial 1,2-CTDs in molecular weight, iron content, optimal pH and metal ion effects. Based on the analyses of MALDI-TOF mass spectrometry and Edman sequencing reported herein, we confirmed that the hypothetical protein CaO19_12036 from *C.albicans* SC5314 is indeed 1,2-CTD.

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