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Regular paper

Characterization of two aminotransferases from *Candida albicans**

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Aminoadipate aminotransferase (AmAA) is an enzyme of α-aminoadipate pathway (AAP) for L-lysine biosynthesis. AmAA may also participated in biosynthesis or degradation of aromatic amino acids and in D-tryptophan based pigment production. The AAP is unique for fungal microorganisms. Enzymes involved in this pathway have specific structures and properties. These features can be used as potential molecular markers. Enzymes catalyzing reactions of L-lysine biosynthesis in *Candida albicans* may also become new targets for antifungal chemotherapy. Search of the NCBI database resulted in identification of two putative aminoadipate aminotransferase genes from *Candida albicans*: *ARO8* (ORFs 19.2098 and 19.9645) and *YER152C* (ORFs 19.1180 and 19.8771). *ARO8* from *C. albicans* exhibits 53% identity to *ARO8* from *S. cerevisiae,* while *YER152C* exhibits 30% identity to *ARO8* and 45% to *YER152C* from *S. cerevisiae*. We amplified two genes from the *C. albicans* genome: *ARO8* and *YER152C*. Both were cloned and expressed as His-tagged fusion proteins in *E. coli*. The purified Aro8CHp gene product revealed aromatic and α-aminoadipate aminotransferase activity. Basic molecular properties of the purified protein were determined. We obtained catalytic parameters of Aro8CHp with aromatic amino acids and aminoadipate (AA) ($K_{m(L\text{-}Phe)}$ 0.05±0.003 mM, $K_{m(L\text{-}Tyr)}$ 0.1±0.008 mM, $K_{m(L\text{-}AA)}$ 0.02±0.006 mM) and confirmed the enzyme broad substrate spectrum. The assays also demonstrated that this enzyme may use 2-oxoadipate and 2-oxoglutarate (2-OG) as amino acceptors. Aro8CHp exhibited pH optima range of 8, which is similar to AmAA from *S. cerevisiae*. Our results also indicate that *Ca*Yer152Cp has a possible role only in aromatic amino acids degradation, in contrast to *Ca*Aro8CHp.

Key words: L-lysine biosynthesis; aminoadipate aminotransferase; aromatic aminotransferase; *Candida albicans*

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INTRODUCTION

L-Lysine is an essential amino acid for humans, while bacteria, plants and fungi have developed pathways of their own lysine biosynthesis. In bacteria, lower fungi (some Phycomycetes) and green plants, L-lysine is synthesized *via* the diaminopimelate pathway in seven steps initiating with aspartate semialdehyde and pyruvate. In Euglenoids and higher fungi (Ascomycetes and Basidomycetes), *de novo* L-lysine biosynthesis proceeds through the intermediacy of L-α-aminoadipate in a series of transformations entirely unrelated to the bacterial diaminopimelate route. Enzymes that catalyze the reaction of the fungal pathway could be a source of new targets for antifungal chemotherapy (Xu *et al*., 2006).

α-Aminoadipate pathway (AAP) of lysine biosynthesis consists of eight stages. Aminoadipate aminotransferase (AmAA) is an enzyme of AAP that catalyzes the fourth reaction: 2-oxoadipate conversion to α-aminoadipate, as shown in Fig. 1.

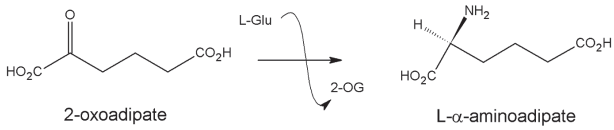

Figure 1. Reaction catalyzed by α-aminoadipate aminotransferase.

Research studies confirm that enzymes exhibiting aminoadipate aminotransferase activity may be involved in several metabolic pathways, e.g. in lysine biosynthesis, Aro8p, Aro9p, Yer152Cp from *S. cerevisiae* and Aro8p, Aro9p, C6_00210W (Yer152Cp) from *C. albicans* (Matsuda & Ogur, 1969; Ghosh *et al.,* 2008) and LysN from *T. termophilus* (*Tht*LysN) (Miyazaki *et al*., 2004), as well as in tryptophan degradation (*Sc*Aro9p) (Urrestarazu *et al*., 1998) and Aro8p and Aro9p from *C. glabrata* (Brunke *et al*., 2010), and in biosynthesis of branched-chain amino acids (*Sc*Aro8p, *Sc*Aro9p and *Tht*LysN) (Miyazaki *et al*., 2004; Urrestarazu *et al*., 1998), and lysine degradation (hKATII from human liver with simultaneous kynurenine aminotransferase activity) (Han *et al*., 2009). Aro8p and Aro9p from *C. glabrata* probably perform the same functions as *Sc*Aro8p and *Sc*Aro9p in respect to aromatic amino acid biosynthesis or degradation. Moreover, their activity is indispensable for pigment production, an important virulence factor of *C. glabrata* (Brunke *et al*., 2010). Similarly, Aro8p and Aro9p from *C. albicans* are essential for aromatic alcohol production (Ghosh *et al.,* 2008). *Sc*Aro9p is able to catalyze the reaction in two directions, although aromatic amino acid and lysine degradation seems to be more likely (Urrestarazu *et al*., 1998).

Aro8p was initially identified as aromatic aminotransferase I, an enzyme involved in the biosynthesis of phenylalanine and tyrosine (Iraqui *et al.,* 1998; Urrestarazu

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Abbreviations: AmAA α-aminoadipate L-α-aminotransferase, AAP α-*aminoadipate* pathway, 2-OG 2-oxoglutarate, PhP phenylpyruvate, 4-hydroxyPhP 4-hydroxyphenylpyruvate, KYNA kynurenic acid, PPA N-phosphopyridoxyl-α-aminoadipate

et al., 1998; Bulfer *et al.,* 2013). Similarly to many class I aminotransferases, Aro8p exhibits a broad substrate specificity and has been reported to utilize glutamate, phenylalanine, tyrosine and tryptophan as amino donors, with phenylpyruvate, 4-hydroxyphenylpyruvate, 2-oxoglutarate and pyruvate as amino acceptors (Kradolfer *et al.,* 1982; Iraqui *et al.,* 1998; Urrestarazu *et al.,* 1998; Karsten *et al.,* 2011). In addition, it can use methionine, leucine, and α-aminoadipate as donors, with their corresponding 2-oxoacids as acceptors. Because *Sc*Aro8p possesses activity towards α-aminoadipate/2-oxoadipate, and *S. cerevisiae* mutant *aro8* strains failed to grow in the presence of a mixture of tyrosine, tryptophan and phenylalanine, it was speculated to have an additional role as one of the glutamate/ 2-oxoadipate transaminases (Iraqui *et al.,* 1998). Further evidence supporting the reclassification of *Sc*Aro8p as an α-aminoadipate aminotransferase derives from a recent study in which this enzyme was shown to display a greater specificity towards 2-oxoadipate compared to the aromatic amino acid substrates, such as phenylalanine and tyrosine (Karsten *et al.,* 2011).

In this paper, we present results of our studies on cloning of two genes *ARO8* (ORFs 19.2098 and 19.9645), and *YER152C,* also known as C6_00210W (ORFs 19.1180 and 19.8771), and characterization of the two Aro8p and Yer152Cp proteins from human pathogenic yeast *Candida albicans*, including their physicochemical properties, substrate specificity, inhibition studies and evidence indicating a different physiological role of both enzymes.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* TOP 10F' strain from Invitrogen was used in all cloning procedures. *E. coli* Rosetta (DE3) pLysS strain from Novagen was used for the overproduction of wild type *Ca*Aro8p, *Ca*Yer152Cp and C-oligoHis-tagged proteins (*Ca*Aro-8CHp, *Ca*YerCHp). *E. coli* strains were cultured at 37°C on LB (Luria–Bertani) solid medium [1.0% (w/v) NaCl, 1.0% (w/v) tryptone, 0.5% yeast extract and 1.5% (w/v) agar] and LB liquid medium supplemented with 100 μg ml^{-1} ampicillin and/or 34 μg ml^{-1} chloramphenicol, when required.

Plasmids, enzymes and other materials. The plasmid used was pET101/D-TOPO (Invitrogen). Restriction enzymes were purchased from Fermentas and New England Biolabs. Protein mass markers were from Thermo Scientific. DNA polymerase was purchased from DNA-Gdansk. Purification of C-oligoHistagged proteins was performed on a HisTrapFF column (GE Healthcare). Purification of native proteins was performed on a ResourceQ column (GE Healthcare). Molecular mass determination was carried out with the Novex NativeP-AGE Bis-Tris gel system (Life Technologies) and with gel filtration using Superdex 200 HR 10/30 GL (GE Healthcare Life Sciences). Monoclonal peroxidase conjugated anti-polyHistidine antibody [HIS-1] and 3, 3', 5, 5'–tetramethylbenzidine, substrates and their analogues, as well as lysine derivatives, were from Sigma. Reagents for determining the activity and inhibition studies were also purchased from Sigma.

DNA manipulations. *Candida albicans* SC5314 genomic DNA was isolated according to the protocol of Bacterial & Yeast Genomic DNA Purification Kit (DNA-Gdansk). Isolation of plasmid DNA was carried out according to the protocol of the Plasmid Mini kit (A&A Biotechnology). DNA fragments were isolated from agarose gels following the standard procedure of the GenElute Gel Extraction Kit (Sigma-Aldrich). DNA digestion with restriction enzymes was carried out according to the enzyme supplier's instructions.

Cloning of the ARO8 and YER152C gene. The fragment of the *ARO8* gene encoding aromatic/aminoadipate aminotransferase was amplified from the *C. albicans* SC5314 genomic DNA by PCR. The primers used in the amplification were: ARO8.f 5'CACCATGACTAGTGATACAAAGCCACAGGCT3'and ARO8CH.r 5'AAACTCGAGCTAAT**GGTGATGGTGATGATGAT**GCAATCCAAACTCGGCTCTGACAG3' for *Ca*Aro8CHp oligoHis-tagged fusion protein and ARO8.f and ARO8.r 5'AAACTCGAGCTACAATCCAAACTCGGCTCTGACAG 3' for the wild type *ARO8* gene cloning. The fragment of *YER152C* gene was amplified with the use of the following primers: YER.f 5'CACCATGATAAACTTCTTCAAGGGCCACC3' and YERCH.r 5'CTAAT**GATGATGATGATGATGAT**GTTCTAAAAGTTCTCCCCAAATC3' for *Ca*YerCHp oligoHis-tagged fusion protein and YER.f and YER.r 5'TAACTATTCTAAAAGTTCTCCCCAAATCTTG3' for the wild type *YER152C* gene cloning. The hexaHis-tag-encoding sequence introduced in the reversed primers are in bold. Primers were designed according to Invitrogen's instructions. The PCR products (1476 bp and 1494 bp for *ARO8*, 1248 bp and 1266 bp for *YER152C,* wild type or with oligo-His constructs, respectively) were purified from an agarose gel and cloned directionally into the pET101/D-TOPO vector giving recombinant expression plasmids: pET101/D-TOPO+*ARO8CH* (7274 bp), pET101/D-TOPO+*ARO8* (7256 bp) and pET101/D-TOPO+*YERCH* (7046 bp), pET101/D-TOPO+*YER152C* (7028 bp). The identity of the plasmids was confirmed by restriction analysis and DNA sequencing. The obtained constructs encoded the putative *Ca*Aro8p and *Ca*Yer152Cp, as well as the same proteins with an additional oligoHis-tag at the C-terminus (*Ca*Aro8CHp and *Ca*YerCHp).

Overexpression of the ARO8 and YER152C genes. *E. coli* Rosetta (DE3) pLysS cells, transformed with the pET101/D-TOPO+*ARO8CH,* pET101/D-TOPO+*ARO8,* and pET101/D-TOPO+*YERCH,* pET101/D-TOPO+*YER152C* expression plasmids, were grown in LB liquid medium with ampicillin and chloramphenicol, overnight at 37°C. Samples of the cultures (10 ml) were then transferred to a fresh LB broth (1 L) containing both antibiotics and grown at 37°C. Expression was induced by the addition of 0.05 mM isopropyl-β-D-thiogalactopyranoside to the cultures grown to OD_{600} 0.4–0.6, and incubation was continued at 30–37°C for another 5–15 h. Cells were harvested by centrifugation at 4000×g for 20 min, at 4°C, and stored for further use at –20°C.

Purification of the oligoHis-tagged Aro8p and Yer152Cp. The C-oligoHis-tagged *Ca*Aro8CHp and *Ca*YerCHp were purified by metal-affinity chromatography. Bacterial pellet was resuspended in buffer A (20 mM Tris-HCl, pH 8, 5 mM imidazole, 0.6 M NaCl and 0.5 mM phenylmethylsulfonyl fluoride /PMSF/) and the cells were disrupted by sonication (5 × 30 s bursts, with 30 s intervals, at a power setting of 30, using a Branson sonifier 250) on ice. The total lysate was centrifuged at 16 000 × *g* for 20 min, at 4°C. The supernatant (crude extract) was applied to a HisTrapFF column, bed volume 5 ml, which was pre-equilibrated with 5 column volumes (CV) of buffer A. Next, the column was washed with 5 CV of the same buffer. The oligoHis-tagged proteins were eluted by increasing concentrations of imidazole

in elution buffer B (20 mM Tris-HCl, pH 8, 500 mM imidazole, 0.5 M NaCl, gradient of buffer A and B: 0–100%). For further assays, the eluates were concentrated by ultrafiltration using Vivaspin concentrators (10 kDa cut-off limit; Viva Science Ltd.) at 7000 × *g* for 30 min, at 4°C, if necessary. The protein preparations were frozen at –20°C in 50 mM phosphate buffer, pH 7.5, with PLP in slight molar excess over the enzyme, and stored for further use.

Purification of the wild type Aro8p and Yer152Cp from *C. albicans*. The supernatant (crude extract) in buffer C (20 mM phosphate buffer, pH 7, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride /PMSF/) obtained after sonication of *E. coli* Rosetta (DE3) pLysS cells, transformed with the pET101/D-TOPO+*ARO8* and pET101/D-TOPO+*YER152C* expression plasmids, was treated with streptomycin sulfate (1.2%) to remove nucleic acids. The resulting mixture was centrifuged (15500 × *g*, 20 min, 4°C). The obtained supernatant was collected for further use at 4°C. Ammonium sulfate was added to 30% saturation, which was found to be the optimal value in order to remove the accompanying proteins with no significant loss of the wild-type *Ca*Aro8p and *Ca*Yer152Cp activity. The suspension was incubated in the ice bath for 20 min and then centrifuged (15500 × *g* for 20 min at 4°C). Supernatant was discarded and the precipitate was dissolved in 10 ml of buffer D (20 mM potassium phosphate buffer, pH 7) with addition of 10 mM $MgCl_2$ and 10 mM NaCl. Proteins were desalted using HiTrap Desalting Columns (GE Healthcare) according to the manufacturer's procedure. The desalted proteins were loaded onto ResourceQ FPLC 6-ml column (GE Healthcare) equilibrated with buffer D. The column was then washed with 20 ml of the same buffer. The wild-type protein was eluted by a gradient of buffer E (buffer D containing 1M NaCl, gradient of buffer D and E: 0–50%). Fractions exhibiting aminotransferase activity were pooled and concentrated by ultrafiltration. Samples were then held frozen (–20°C) in 50 mM phosphate buffer pH 7.5, with PLP in slight molar excess over enzyme and stored for further use.

Western-Blott analysis. Proteins were separated on 15% polyacrylamide gel according to Laemmli (Laemmli, 1970). Electrophoresis was followed by electroblotting to a nitrocellulose membrane with the use of a transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol). Nitrocellulose membranes were incubated in 3% skimmed milk solution, in washing buffer: 10 mM Tris-HCl, pH 8.0, 30 mM NaCl, for 1h. After three time washing with washing buffer, membranes were incubated for 1h with monoclonal peroxidase conjugated anti-poly-Histidine antibody [HIS-1] 1:2000 solution. *Ca*Aro8CHp or *Ca*YerCHp were detected by the addition of peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) according to manufacturer's instructions (Sigma).

Molecular-mass determination of the native protein. Size-exclusion chromatography was performed on Superdex 200 HR 10/30, proteins were eluted at a flow rate of 0.5 ml min^{-1} with 25 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Protein elution was monitored at 280 nm. The molecular-mass standards were: *a*-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrom *c* (12.4 kDa). Oligomeric structure was also analyzed by native PAGE electrophoresis using the NativePAG Novex 4–16% Bis-Tris Gels kit (Invitrogen). The experiments were run according to the manufacturer's procedure.

Kinetic measurements and substrate specificity studies. In all assays, 2.5 μg of purified enzyme was used.

Aromatic amino acid degradation. The concentration of amino group donor (L-Phe, L-Tyr, L-Trp) was 0.006–10 mM, and amino group acceptor was 0.25–20 mM (2-oxoglutarate, pyruvate, gloxylate, 2-oxoadipate). The reaction was carried out in 100 mM Tis-HCl pH 7.5 with 0.03 mM of PLP, in the final volume of 200 μl. The reaction mixtures containing all the required components except the enzyme, were preincubated for about 3 min and the reaction was started by an addition of the enzyme to the mixture and run for 2 min at room temperature (RT). This time period was chosen as optimal for determination of initial reaction rates. The reaction was then terminated by addition of 800 μl of 2.5 M NaOH. The product formation was measured spectrophotometrically with the use of Spectrophotometer UV-Vis PerkinElmer Lambda 45 6K15 (320 nm for phenylpyruvate (PhP); ε 17 700 dm^3 cm^{-1} mol^{-1}, 331 nm for 3-indolepyruvate; ε 19 900 dm^3 cm^{-1} mol^{-1} and 338 nm for 4-hydroxyphenylpyruvate (4-hydroxyPhP); ε 9 300 dm^3 cm^{-1} mol^{-1}).

Aromatic amino acids biosynthesis. The concentration of amino group donor (L-Glu) was 1.5-45 mM and amino group acceptor was 0.005–0.06 mM (PhP, 4-hydroxyPhP). The reaction was carried out in 100 mM Tis-HCl pH 7.5 with 0.03 mM of PLP, in the final volume of 200 μl, and started by the addition of the enzyme as mentioned above. After 2 min of incubation, 800 μl of 2.5 M NaOH was added. The level of product formation, Phe or Tyr, was estimated by a spectrophotometrically measured decrease in the PhP and 4-hydroxyPhP absorbance, respectively (320 nm for PhP; ε 17 700 dm^3 cm^{-1} mol^{-1} and 338 nm for 4-hydroxyPhP; ε 9 300 dm^3 cm^{-1} mol^{-1}).

α-Aminoadipate biosynthesis. The concentration of L-glutamate used was 2–20 mM, and 2-oxoadipate was at 0.2–1.5 mM. The reaction was carried out under the same conditions as mentioned above, and run for 5 min at RT. This time period was chosen as optimal for determination of initial reaction rates. The reaction was then terminated by addition of 60 μl of 1 M HCl. Then, 60 μl of 1 M NaOH was added for neutralization. 100 μl of the reaction mixture was added to 750 μl of 20 mM phosphate buffer pH 7 containing 0.05 M NH_4Cl, 0.1 mM NADH and 2.5 U of L-glutamic dehydrogenase. Formation of NAD^+ was measured spectrophotometrically after 30 min of incubation in RT (340 nm; ε 6 200 dm^3 cm^{-1} mol^{-1}).

α-Aminoadipate degradation. The concentration of α-aminoadipate used was 0.01–0.2 mM, and 2-oxoglutarate was at 0.1–5 mM. The reaction was carried out under the same conditions as mentioned above, and run for 2 min at RT. The reaction was then terminated by incubation at 80°C for 5 min. After cooling on ice, 20 μl of the reaction mixture was used for determining the amount of the reaction product. The formation of glutamate was measured spectrophotometrically with the use of L-Glutamate Assay Kit (Sigma) according to the manufacturer's instructions.

Aro8CHp inhibition studies. The effect of different compounds on Aro8CHp activity was determined by measuring the enzyme activity in standard assay mixtures containing various concentrations of 2-OG analogues: oxalate, glyoxylate, oxobutyrate, and amino donor group analogues like: L-canavanine, L-norleucine, L-lysine, diaminopimelate, pipecolate.

Determination of optimal pH. Determination of optimal pH for the enzyme was done by measuring activity as described above with changing pH of the assay buffer, ranging from 6 to 9.

Other methods. Protein concentration was determined by the Bradford method (Bradford 1976). Discontinuous SDS/PAGE was performed by the method of Laemmli (Laemmli, 1970) using a 12% (w/v) separating gel and a 5% (w/v) stacking gel.

RESULTS AND DISCUSSION

It has been known that in *S. cerevisiae* open reading frames, YGL202W (*ARO8*) and *YER152C* encode enzymes that harbor α-aminoadipate aminotransferase activity (King *et al.*, 2009). BLAST search using the nucleotide sequence of the respective ORFs from the *Saccharomyces* Genome Database performed on the *Candida* Genome Database revealed 19.2098 and 19.9645 ORFs named *ARO8/C2_00340C,* and 19.1180 and 19.8771 named C6_00210W, highly homologous to their *S. cerevisiae* counterparts.

The *ARO8/C2_00340C* ORFs from *Candida albicans* contain 1476 nucleotides and code for a 491 aa protein (*Ca*Aro8p), while C6_00210W ORFs consist of 1248 nucleotides encoding a 415 aa protein (*Ca*Yer152Cp). The deduced amino acid sequences of *Ca*Aro8p and *Ca*Yer152Cp are highly homologous to each other (24% identity, 44% similarity). The amino acid sequence of a putative *CaARO8* product shows 53% identity to that of Aro8p from *S. cerevisiae* (*Sc*Aro8p,) respectively, and 23–31% to AmAA from *Homo sapiens, Rattus norvegicus, Mus musculus, Bos taurus* or *Thermus thermophilus*. *CaYER152C* gene product shows 45% identity to *Sc*Yer152Cp and 25-29% to AmAA from *Homo sapiens, Rattus norvegicus, Mus musculus, Bos taurus, Thermus thermophilus*. Multiple sequence alignment (Fig. 2) and amino acid sequence analysis revealed the presence of conserved residues.

Cloning and expression of the *CaARO8* and *CaYER152C* genes

The *CaARO8* and *CaYER152C* genes were cloned into the expression vector pET101/D-TOPO in a system that enabled overproduction of recombinant wild type proteins or proteins containing the C-terminal oligoHis-tag sequence. The PCR fragments amplified by using the indicated primers (ARO8.r, ARO8CH.r and ARO8.f for *ARO8,* and YER.r, YERCH.r and YER.f for *YER152C*) were cloned into the pET101/D-TOPO expression plasmid according to Invitrogen's procedure. Resulting pET101/D-TOPO+*ARO8*, pET101/D-TOPO+*ARO8CH* and pET101/D-TOPO+*YER152C*, pET101/D-TOPO+*YERCH* plasmids were sequenced. The expression plasmids, in which the *ARO8* or *YER152C* genes are placed under the control of a T7 promoter, were transferred into *E. coli* Rosetta (DE3) pLysS strain. Overexpression of the cloned genes, induced upon IPTG addition resulted in a production of soluble wild type and oligoHis-tagged proteins in *E. coli* cells.

Purification of CaAro8p and CaYer152Cp

The purification of recombinant *Ca*Aro8p or *Ca*Yer152Cp was achieved in several steps including streptomycin sulfate, ammonium sulfate precipitation and ion exchange chromatography. Fractions eluted from the ResourceQ column by NaCl gradient were analyzed by SDS–PAGE (Fig. 3). Densitometric analysis shows ≥91% homogeneity of *Ca*Aro8p fraction, and ≥61% homogeneity of *Ca*Yer152Cp fraction (GelQuant Pro).

Purification of CaAro8CHp and CaYerCHp

The purification of recombinant *Ca*Aro8CHp or *Ca*YerCHp was achieved in a single chromatographic step with the use of HisTrapFF column. Fractions eluted from the column by an imidazole gradient were analyzed by SDS–PAGE (Fig. 4A) and Western blotting (Fig. 4B). The recombinant proteins were purified to near homogeneity (≥98% for *Ca*Aro8CHp) or with purity sufficient for kinetic analysis (≥74% for *Ca*YerCHp), as revealed by densitometry (GelQuant Pro). Western blotting analysis confirmed presence of the oligoHis-tagged fusion proteins in the appropriate fractions.

Characterization of *Ca*Aro8p and *Ca*YER152Cp molecular and catalytic properties

Molecular mass

Results of the SDS-PAGE analysis indicated MW of the *Ca*Aro8CHp subunit equal to 55 ± 9062 kDa and that of the *Ca*YerCHp to 48 ± 2kDa, in agreement with the theoretical values of 56.4 kDa and 47.3 kDa, respectively. Molecular subunit composition and MWs of native forms of both recombinant proteins were estimated using the size exclusion chromatography (SEC) and blue native gel analysis. Peak present in the *Ca*Aro8CHp SEC profiles eluted at volume corresponding to MW 73.5 ± 8 kDa. Results obtained by electrophoresis under non-denaturing conditions revealed the presence of two protein bands corresponding to the molecular masses of 69.5 ± 10.5 kDa and 141.5 ± 40 kDa. Results of both analyses revealed that *Ca*Aro8p may exist as a monomer, although the results obtained by blue native gel analysis seem to indicate the presence of dimeric forms. Crystal structures of enzymes exhibiting mainly aromatic aminotransferase activity from *S. cerevisiae* (*Sc*Aro8p), putative α-aminoadipate aminotransferase (Bulfer *et al.*, 2013) and α-aminoadipate aminotransferase from *Thermus thermophilus* (*Tht*LysN) (Tomita *et al.*, 2009) revealed that both exist as homodimers. Human kynurenine aminotransferase II (hKATII) that notably catalyzes the synthesis of kynurenic acid (KYNA) but is unique in having α-aminoadipate aminotransferase activity is also supposed to be a homodimer (Han *et al.*, 2009).

The same results were obtained for *Ca*YerCHp. Peak present in the SEC profiles eluted at a volume corresponding to MW 63 ± 6 kDa. Results obtained by electrophoresis under non- denaturing conditions revealed the presence of two protein bands, corresponding to the molecular masses of 55 ± 8 kDa and 97 ± 27 kDa. Results of both analyses suggested a monomeric or dimeric structure of *Ca*YerCHp, although it is unusual for aminotransferases to form monomers. For example, alanine aminotransferases from *P. furious* and *C. maltosa* are dimers (Ward *et al.*, 2000; Umemura *et al.,* 1994). The same oligomeric structures are observed for both, asparte aminotransferases from *B. subtilis* or *E. coli* (Sung *et al.*, 1990; Smith *et al.*, 1989), as well as for aromatic aminotransferases from *S. cerevisiae* (Bulfer *et al.*, 2013). As far as we know, our results concerning oligomeric structure of *Ca*YerCHp are reported for the first time for Yer152Cp from any organism.

A

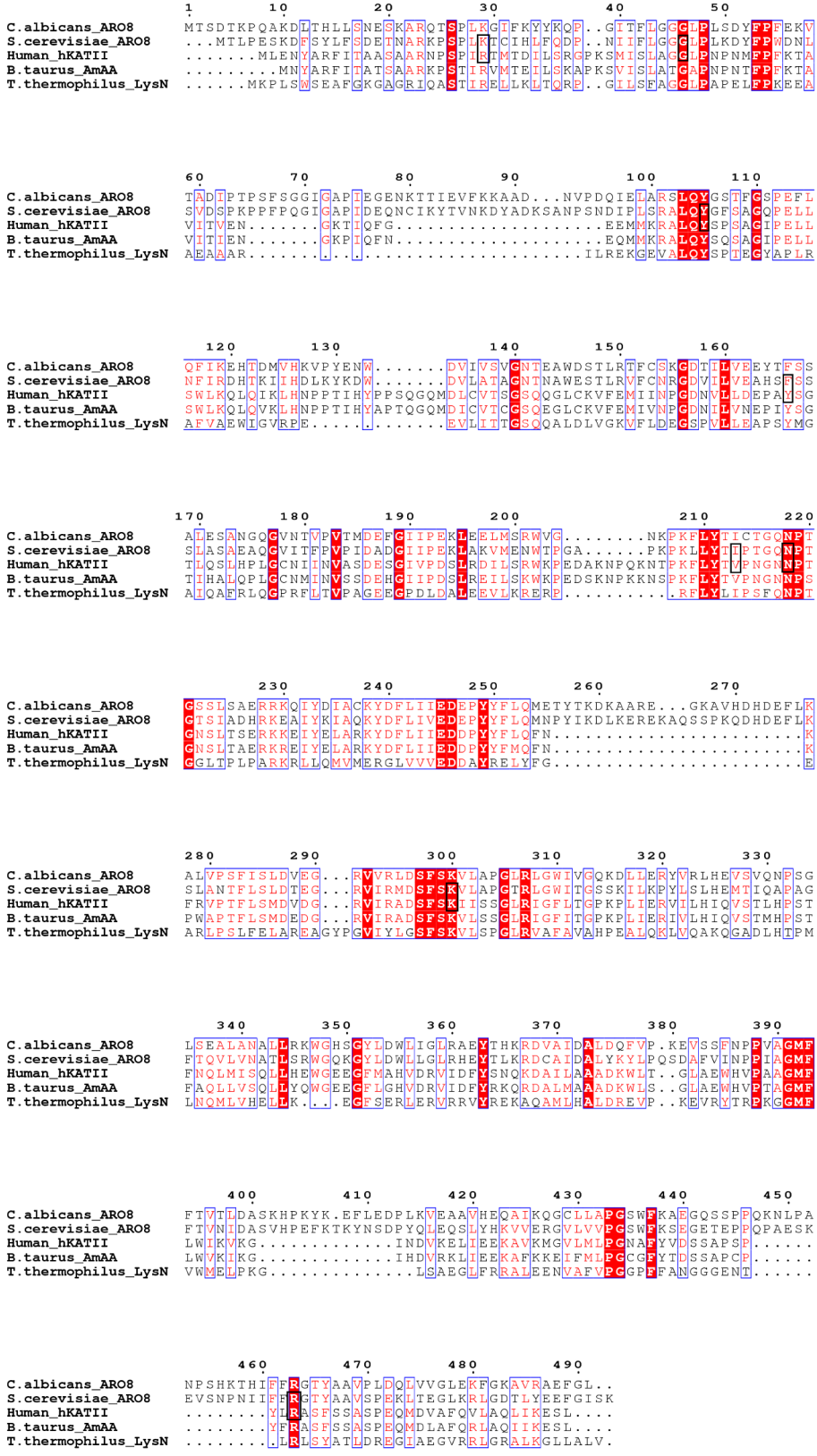

Figure 2 Multiple amino acid sequence aligment for (A) *Ca*Aro8p and (B) *Ca*Yer152Cp from *Candida albicans* and aminotransferases from other organisms.
Highly conserved residues are marked as white letters on a red background, similarities are marked in red, the most important residues participating in substrate and PLP binding in hKATII and *Sc*Aro8p are marked in bold black frame (Rossi *et al*., 2008; Han *et al*., 2009; Bulfer *et al*., 2013). ClustalW (Larkin *et al*., 2007) was used to perform a multiple sequence alignment and ESPript (Robert & Gouet 2014) was used to produce the figure.

B

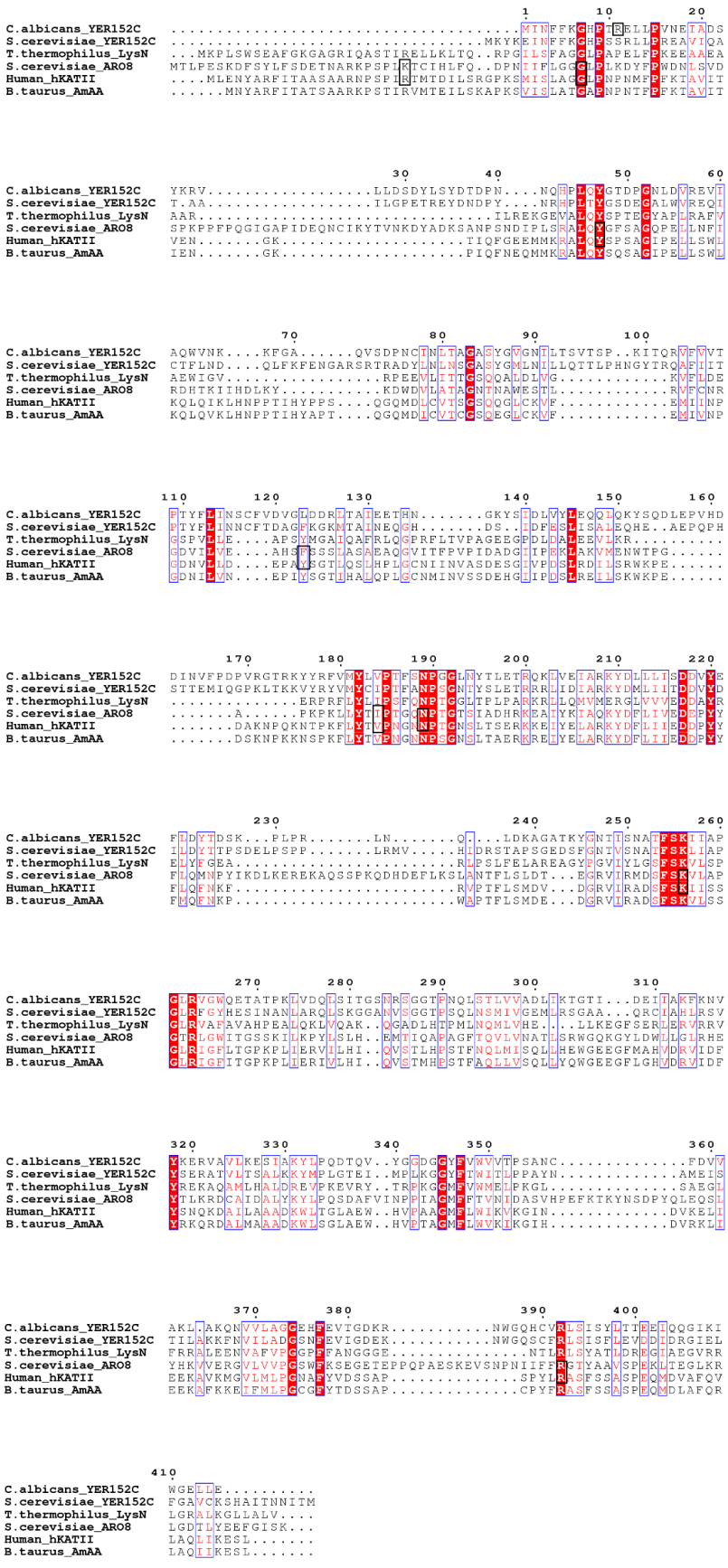

Figure 2. Continued

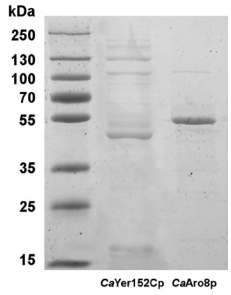

Figure 3. SDS-PAGE of the final purified fractions of *Ca*Yer152Cp and *Ca*Aro8p form *C. albicans*.

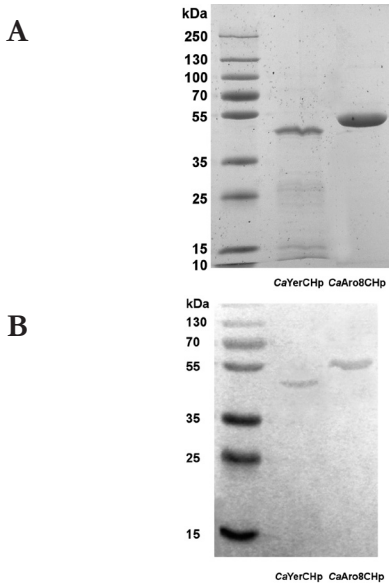

Figure 4. SDS-PAGE (A) and Western blotting (B) analysis of the final purified fractions of C-terminally His_6-tagged *Ca*YerCHp and *Ca*Aro8CHp from *C. albicans,* overproduced in *E. coli*.

Kinetic properties and substrate specificity

To characterize both *ARO8* and *YER152C* gene products in detail, kinetic analysis was carried out with purified oligoHis-tagged proteins and compared to their wild type counterparts. To understand the role of *Ca*Aro8p and *Ca*Yer152Cp, a screen of several potential substrates for the aromatic and/or aminoadipate aminotransferase was undertaken. *Ca*Aro8p showed transamination of PhP and 4-hydroxyPhP as the amino acceptors, and L-Glu as the amino donor to produce phenylalanine and tyrosine, respectively. Similar analysis was done in the reverse reaction, at the same pH, with L-Phe or L-Tyr and 2-oxoglutarate as the amino donor and the amino acceptor, respectively, to determine K_m values (Table 1). From this analysis, we concluded that *Ca*Aro8p is mainly a biosynthetic enzyme devoted to aromatic amino acid biosynthesis. The enzyme was shown to display greater affinity towards PhP and 4-hydroxyPhP than Phe or Tyr.

Our results are similar to those obtained for a corresponding enzyme from *S. cerevisiae*, which was initially identified as an aromatic aminotransferase I (*Sc*Aro8p), an enzyme involved in the biosynthesis of phenylalanine and tyrosine (Kradolfer *et al.*, 1982). Those authors postulated that *Sc*Aro8p plays an essentially biosynthetic role, being the only aromatic aminotransferase present in cells grown on minimal medium containing ammonium ions as a sole nitrogen source.

Table 1. Comparison of *Ca*Aro8CHp kinetic parameters against substrates for biosynthesis or degradation of aromatic amino acids and α-aminoadipate.

Substrate	K_m [mM]	V_{max} [nmol min^{-1} mg^{-1}]
Amino group acceptor: 2-oxoglutarate		
L-Phe	0.05 ± 0.003	1217 ± 29
L-Tyr	0.1 ± 0.008	1706 ± 41
L-Trp	1.45 ± 0.164	6308 ± 230
L-AA	0.02 ± 0.006	856 ± 57
Amino group donor: L-Glu		
PhP	0.01 ± 0.002	630 ± 35
4-hydroxyPhP	0.03 ± 0.003	909 ± 38
2-oxoadipate	0.1 ± 0.033	9954 ± 2032

We next analyzed the transamination reaction with several 2-oxoacids as the amino acceptors by using phenylalanine as an amino donor (Table 2).

Our results indicate a broad spectrum specificity of *Ca*Aro8p. Furthermore, *Ca*Aro8p was able to utilize L-Glu as an amino group donor and 2-oxoadipate as an amino group acceptor to produce α-aminoadipate. Our results indicate that aromatic aminotransferase I (*Ca*Aro8p) from *C. albicans* plays an important role in L-lysine biosynthesis as an α-aminoadipate aminotransferase. In addition, K_m value determined for L-Glu and 2-oxoadipate was one order of magnitude greater than that obtained for L-Glu and phenylpuryvate, or L-Glu and 4-hydroxyphenylpuryvate (Table 1).

The ability of the *ARO8* encoded aminotransferase to utilize both, aromatic and dicarboxylic acid substrates, appears to be a common feature of this class of aminotransferase enzymes. The aromatic aminotransferase from *Paracoccus dentrificans* (*Pd*Aro8p) is an example. This aminotransferase can use both, acidic and aromatic amino acids as substrates (Okamoto *et al.*, 1998). *Pd*Aro8p has the ability to switch its recognition site from a carboxylate side chain to an aromatic side chain by rearranging a set of active site residues to accommodate the aromatic amino acid. The human α-aminoadipate/L-kynurenine aminotransferase II (hKAT II) has been reported to use 16 amino acids as substrates, with a wide range of side chains and a similarly wide range of 2-oxoacids as amino group acceptors (Han *et al.*, 2009). The crystal structure of the human enzyme hKATII in complex with L-kynurenine reveals amino acid residues in the active site that are in contact with L-kynurenine (Fig. 2A). Five of these residues are conserved in the enzyme from *S. cerevisiae* (Gly43', Tyr105', Asn220, Arg470, Lys305) and the remaining three are conservatively substituted (Tyr142, Val199 and Arg20 in hKATII for Phe166, Ile215 and Lys26 in *Sc*Aro8p, respectively). The pocket that accommodates the aromatic side chain of L-kynurenine in the

Table 2. Comparison of *Ca*Aro8CHp affinity against various 2-oxoacids.

Substrate	K_m [mM]	V_{max} [nmol min^{-1} mg^{-1}]
Amino group donor: L-Phe		
2-Oxoadipate	0.81 ± 0.09	11796 ± 534
Pyruvate	3.59 ± 0.54	1895 ± 120
Glyoxylic	7.04 ± 0.65	3458 ± 115
2-Oxoglutarate	5.19 ± 0.71	3618 ± 209

human enzyme could potentially accommodate the aliphatic portion of a substrate, such as L-α-aminoadipate. Karsten *et al.* suggests that similar interactions could be involved in the yeast enzyme (Karsten *et al.*, 2011). Data obtained from a crystal structure of *Sc*Aro8p (Bulfer *et al.*, 2013) indicate that four of these residues, Gly43', Asn220, Arg470 and Tyr105' are mainly involved in different substrate binding. Those residues are also highly conserved in *Tht*LysN, hKATII and *Bos taurus,* and are involved in the catalysis (Han *et al.*, 2009; Tomita *et al.*, 2009; Ouchi *et al.*, 2009). The corresponding residues are also present in the *Ca*Aro8p and *Ca*Yer152Cp amino acid sequences (Gly46', Asn218, Arg463 and Tyr105' from *Ca*Aro8p, and Gly7', Asn189, Arg392 and Tyr47' from *Ca*Yer152Cp). Multiple sequence alignment analysis also shows other conserved residues among species (Fig. 2A). The eight most important residues participating in substrate and PLP binding to hKATII are identical or conservatively substituted in *Ca*Aro8p. There are only 3 differences between these residues comparing to eight most important residues from hKATII and there is no difference comparing to *Sc*Aro8p. There is a change in hKATII Arg20 to Lys29 in *Ca*Aro8p (similarly in *Sc*Aro8p there is Lys26). This substitution may be significant for the enzyme affinity against substrates. It has been reported that modification of Arg23 residue in LysN from *T. thermophilus* (corresponding to Arg20 residue in hKATII) decreases the catalytic efficiency for glutamate as a substrate (Ouchi *et al.*, 2009). N-phosphopyridoxyl-α-aminoadipate (PPA) and kynurenine substrates in *Tht*LysN and hKATII, respectively, are also recognized by a conserved arginine residue (Arg23 in *Tht*LysN and Arg20 in hKATII). In *Tht*LysN, the δ-carboxylate group of the aminoadipate moiety of PPA is stabilized through salt bridge interactions with the guanidinium group of Arg23 in *Tht*LysN (Tomita *et al.*, 2009), and the corresponding residue in hKATII, Arg20, participates in cation-π stacking with the kynurenine substrate (Rossi *et al.*, 2008; Han *et al.*, 2009). As was mentioned above, in *Sc*Aro8p, this arginine is substituted with Lys26, and this lysine residue may rearrange to play an important role in substrate recognition, analogous to the corresponding arginine residue in *Tht*LysN and hKATII. A model of kynurenine bound to the active site of *Sc*Aro8p displays similarities to the kynurenine-bound structure of hKATII indicating that enzyme from *S. cerevisiae* may be able to process this substrate despite the presence of Lys26. Our preliminary studies confirmed that despite the occurrence of an analogous lysine residue, Lys29 in *C. albicans* Aro8p sequence, *Ca*Aro8p is able to catalyze kynurenine transamination (data not shown).

Another difference is observed for the Tyr142 residue from hKATII. In *Ca*Aro8p, the Tyr residue is replaced by Phe166. Similar change is observed for several AmAA, like *Sc*Aro8p, hKATI and AmAA from *Mus musculus*. It is known that Tyr142 from hKATII participated in PLP binding, nevertheless more often Phe or Trp fulfills that function (Rossi *et al.*, 2008). PLP forms a Schiff base with the α-amino group of Lys305 from *Sc*Aro8p (Bulfer *et al.*, 2013). This residue is highly conserved among species and is also present in the *Ca*Aro8p and *Ca*Yer152Cp amino acid sequences (Lys300 and Lys256, respectively). Based on this observation and amino acid sequence alignment, substrates of *Ca*Aro8p and *Ca*Yer152Cp are predicted to bind through similar interactions with these residues.

According to a previous report suggesting that Yer-152Cp from *S. cerevisiae* has an aminoadipate aminotransferase activity (King *et al.*, 2009), a corresponding gene product from *C. albicans* was analyzed. A screen of several potential substrates for the *Ca*Yer152Cp and *Ca*YerCHp was undertaken. Although amino acid sequence alignment indicates the presence of amino acids that are the most important for catalysis and substrate binding, and suggests the same mode of enzyme activity under the conditions used by us, *Ca*YerCHp showed neither aromatic nor aminoadipate transamination activity. Wild type *Ca*Yer152Cp was able to catalyze only the conversion of L-Phe or L-Tyr and 2-oxoglutarate, as the amino donors and the amino acceptor, respectively. The determined K_m values for that reaction indicate a four-fold worse affinity against L-Phe and L-Tyr for *Ca*Yer152Cp, when comparing to *Ca*Aro8CHp (Table 3). The specific activity is one order of magnitude lower for each aromatic amino acid. When similar analysis was done in the reverse reaction, at the same pH, with PhP and 4-hydroxyPhP as the amino acceptors, and L-Glu as the amino donor, to produce phenylalanine and tyrosine, respectively, no activity was detected.

Table 3. Comparison of *Ca*Yer152Cp kinetic parameters against substrates for degradation of aromatic amino acids.

Substrate	K_m [mM]	V_{max} [nmol min^{-1} mg^{-1}]
Amino group acceptor: 2-oxoglutarate		
L-Phe	0.24 ± 0.024	184 ± 4
L-Tyr	0.46 ± 0.122	164 ± 15
L-Trp	1.07 ± 0.182	383 ± 16

Our results indicate that *Ca*Yer152Cp has a possible role in aromatic amino acid degradation. This narrow substrate spectrum is surprising due to the sequences similarity of the most important residues. Multiple sequence alignment shows that *Ca*Yer152Cp has six identical residues in the active site, as does hKATII (Fig. 2B). There are only two differences in the eight most important residues participating in substrate and PLP binding to hKATII. Val199 from hKATII is substituted by Ile184 (similar to *Sc*ARO8p Ile215), Tyr142 is replaced by Leu124. Despite such small differences, Tyr142 substitution for Leu124 seems to be crucial for the enzyme's activity and substrate recognition. It has been reported that Tyr142 together with Asn202, Gly39' and Tyr74' define the hKATII binding-site (Han *et al.*, 2009). Tyr142 in hKATII substituted for Phe125 in hKATI determined differences in substrate recognition (Rossi *et al.*, 2008). All aminotransferases being compared (Fig. 2), show a wide substrate spectrum and have corresponding aromatic residues (Phe or Tyr). Although only in the case of *Ca*Yer152Cp's amino acid sequence there is a lack of equivalent Tyr or Phe residue, the assumption of Leu124 having a crucial role needs further analysis.

N-phosphopyridoxyl-α-aminoadipate (PPA) and kynurenine substrates in *Tht*LysN and hKAT II, respectively, are also recognized by a conserved arginine residue (Arg23 in *Tht*LysN, and Arg20 in hKAT II) (Tomita *et al.*, 2009). In *Ca*Yer152Cp there is an Arg11 (Fig. 2B) that may correspond to the Arg23 in *Tht*LysN, and Arg20 in hKATII. Preliminary results of *Ca*Yer152Cp activity with kynureine as a substrate, confirmed that the enzyme from *C. albicans* is able to catalyze kynurenine transamination (data not shown).

The effect of pH on enzyme activity

To analyze whether there are some differences concerning the optimal pH for the reactions catalyzed by

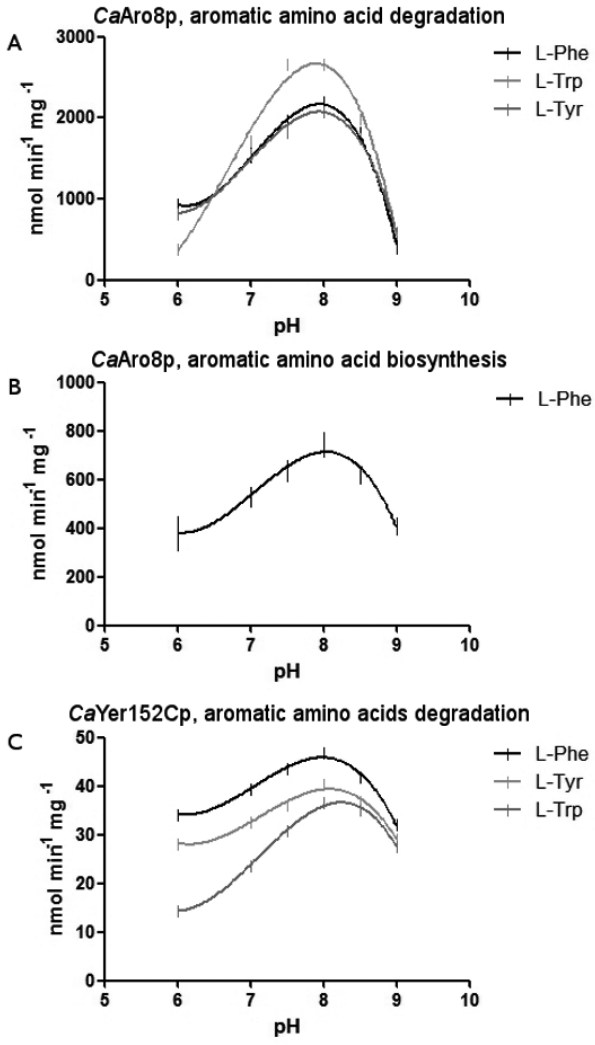

Figure 5. Comparison of the "enzymes" specific activity at different pH.
(**A**) *Ca*Aro8CHp degradation of aromatic amino acids, (**B**) *Ca*Aro-8CHp synthesis of L-Phe, (**C**) *Ca*Yer152Cp degradation of aromatic amino acids.

*Ca*Aro8CHp and *Ca*Yer152Cp, we analyzed the effect of pH on the enzyme activity (Fig. 5). The estimated optimal pH for both, *Ca*Aro8CHp and *Ca*Yer152Cp, was pH 8. The same results were obtained regardless of the reaction analyzed, neither aromatic amino acids degradation nor biosynthesis was differentially affected by pH. Similar optimal pH was observed for *Sc*Aro8p (pH 8.5) (Matsuda & Ogur, 1969).

*Ca*Aro8CHp inhibition studies

Due to the potential role of the enzyme with α-aminoadipate aminotransferase activity as an antifungal drug target, inhibition analyses were carried out. In our present studies, we analyzed the influence of several 2-OG and 2-oxoacid analogues on Aro8CHp activity. Compounds were selected according to the lack of C2-oxo group and the length of chain (dicarboxylic acids, such as glutarate, succinic acid and oxalate) or the lack of carboxyl group corresponding to the following groups at the C5 position of 2-oxoglutarate (2-oxoacids like glyoxylic and 2-oxobutyric acid). According to the previous reports concerning inhibition of murine kynurenine aminotransferases by some proteinogenic amino acids (Han *et al*., 2010), the effect of selected amino acids on Aro8CHp activity was also analyzed. Aro8CHp was not inhibited by the end product of the α-aminoadipate pathway, L-lysine and its derivative, L-canavanine. On the other hand, moderate inhibition of Aro8CHp was observed for L-norleucine and oxalic acid, with IC_{50} 2.8 ± 1.31 mM and 6.3 ± 1.01 mM, respectively. There was neither inhibitory effect for diaminopimelic acid, a precursor in L-lysine biosynthesis in bacteria, nor for pipecolic acid-amino acid analogue. The same situation was observed for oxobutyric acid and glyoxylic acid. Both were recognized by *Ca*Aro8p as substrates.

In conclusion, two *ARO8* and *YER152C* genes of *Candida albicans* were unequivocally identified as encoding aminotransferases. The genes were cloned, sequenced, and overexpressed in *Escherichia coli,* as wild type and fusion proteins with C-terminal oligoHis-tag. In this respect, it is worth mentioning that as far as we know, our results concerning the physicochemical properties, substrate specificity, inhibition studies and evidence provided for physiological role of *Ca*Yer152Cp, comprise the first report ever for Yer152Cp from any organism. *Ca*-Aro8p is the first aromatic/aminoadipate aminotransferase from human pathogenic fungi that was isolated and characterized. This enzyme's essential role in aromatic amino acid and lysine biosynthesis was identified. Moreover, *Ca*Aro8p exhibited the ability to catalyze the reverse reaction, the aromatic amino acid degradation. On the other hand, despite high degree of similarity in amino acid sequence, *Ca*Yer152Cp is able to catalyze only the conversion of L-Phe or L-Tyr and 2-oxoglutarate, as the amino donors and the amino acceptor, respectively. Our results indicate that *Ca*Yer152Cp has a possible role in aromatic amino acid degradation.

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