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Experimental and bioinformatic investigation of the proteolytic degradation of the C-terminal domain of a fungal tyrosinase

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ABSTRACT

Proteolytic processing is a key step in the production of polyphenol oxidases such as tyrosinases, converting the inactive proenzyme to an active form. In general, the fungal tyrosinase gene codes for a ~60 kDa protein that is, however, isolated as an active enzyme of ~40 kDa, lacking the C-terminal domain. Using the secreted tyrosinase 2 from *Trichoderma reesei* as a model protein, we performed a mutagenesis study of the residues in proximity of the experimentally determined cleavage site which are possibly involved in the proteolytic process. However, the mutant forms of tyrosinase 2 were not secreted in a full-length form retaining the C-terminal domain, but they were processed to give a ~45 kDa active form. Aiming at explaining this phenomenon, we analysed *in silico* the properties of the C-terminal domain of tyrosinase 2, of 23 previously retrieved homologous tyrosinase sequences from fungi (C. Gasparetti, G. Faccio, M. Arvas, J. Buchert, M. Saloheimo, K. Kruus, Appl. Microbiol. Biotechnol. 86 (2010) 213–226) and of nine well-characterised polyphenol oxidases. Based on the results of our study, we exclude the key role of specific amino acids at the cleavage site in the proteolytic process and report an overall higher sensitivity to proteolysis of the linker region and of the whole C-terminal domain of fungal tyrosinases.

1. Introduction

Proteolytic processing is a post-translational modification involved in the maturation of different proteins. Among these, polyphenol oxidases such as tyrosinases from fungi are usually produced in a truncated form lacking the C-terminal ~20 kDa polypeptide sequence also referred to as C-terminal domain. Since tyrosinases, laccase and catechol oxidases share common phenolic substrates, they are generally grouped under the term "polyphenol oxidase". However, only tyrosinases oxidise mono-phenolic compounds, e.g. tyrosine. Most studied tyrosinases such as the commercial enzyme from the common button mushroom *Agaricus bisporus* [1–4], from the bread mould *Neurospora crassa* [5] and the secreted fungal tyrosinase from the cellulolytic fungus *Trichoderma reesei* [6] are C-terminally processed. As a typical case, the gene of tyrosinase 2 from *T. reesei* coded for a 61.5 kDa protein. The mature secreted tyrosinase had a significantly lower molecular mass of 43.2 kDa when overexpressed in the native host [6]. The commercially available tyrosinase from *A. bisporus* is produced as an active 44 kDa protein although the gene codes for a full-length 63.8 kDa inactive protein [2,3,7]. Structurally similar enzymes such as catechol oxidase from sweet potato *Ipomoea batatas* [8], polyphenol oxidases from broad bean *Vicia faba* and grapes *Vitis vinifera* [9,10] are subject to analogous processing for maturation. In some cases, the full-length form with a molecular mass of ~60 kDa has been isolated and it was reported to undergo activation either upon removal of the C-terminal domain by proteolysis [11–13] or, in a reversible manner, by loosening of the structure, e.g. after denaturing agents and detergents were used [14–18]. The function of the C-terminal domain is debated and it has been ascribed to form a shield over the active site and maintain the enzyme inactive [11,12,19]. Polyphenol oxidases are structurally related to the oxygen-binding proteins haemocyanins that, interestingly, do not naturally undergo proteolytic processing but acquire polyphenol oxidase activity upon proteolytic treatment [20].

Tyrosinases are copper-dependent enzymes able to oxidise mono- and di-phenolic molecules to the corresponding *ortho*-quinones. Tyrosinases catalyse the first step of the melanin biosynthesis pathway by oxidising L-DOPA (L-3,4-dihydroxyphenylalanine) or tyrosine [21] that undergo subsequent non-enzymatic polymerisation. Like catechol oxidases and haemocyanins, tyrosinases belong to the class of type-3 copper proteins. These are characterised by the presence of two triads of histidine residues coordinating the two copper ions of the active site (CuA and CuB). Many studies, both experimental and *in silico*, have identified the residues that are involved in the folding and function of the enzymes [22]. Two sequence motifs have been identified in the proximity of the cleavage region between the core domain and the C-terminal domain of fungal tyrosinases (Fig. 1). While the first motif, the tyrosine motif (Y-motif) [22,23], is highly conserved and it interacts with the N-terminal extremity of the

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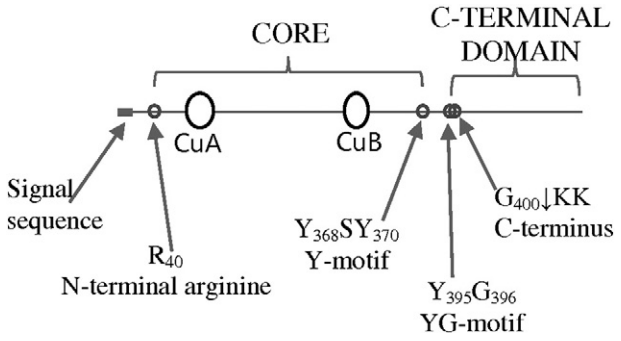

Fig. 1. Sequence motifs and residues identified in tyrosinases. Distances and residue positions are related to the tyrosinase 2 from *T. reesei*. The 18-residue-long signal sequence is reported as a thick line. The core domain is delimited by the N-terminal arginine and the C-terminal tyrosine motif [25]. The YG motif is retained in the mature enzyme and its role has not been established yet [6,13,22]. The copper binding motifs A (CuA, H_{81}–X_{21}–C_{103}–P–H_{105}–X_8–H_{114}) and B (CuB, H_{271}–X_3–H_{275}–X_{24}–H_{300}) are indicated.

globular core in the three-dimensional structure [8,24], the second motif is the tyrosine-glycine motif (YG-motif) that is not always present and whose role is not clear [22]. The YG-motif is especially found in fungal polyphenol oxidases [22].

The active core domain of polyphenol oxidases is characterised by the presence of highly conserved residues such as a histidine pattern for cofactor binding and additional aromatic residues [23,25]. On the other hand, the C-terminal protein region following the Y-motif shows a significantly lower level of homology and its length significantly varies among proteins of different origin. To our knowledge, no study has reported the isolation of the C-terminal domain in a stable form and its characterisation. Although the three-dimensional structures of five tyrosinase and catechol oxidase have been solved, no structure containing the C-terminal domain could be obtained [26–30]. However, tyrosinase from *A. bisporus* was crystallised in a complex with a second, smaller protein that had a lectin-like structure [26], and the bacterial tyrosinase from *Streptomyces castaneoglobisporus* in complex with a so-called caddie protein ORF378 [31]. Previously, Inlow et al. have analysed the sequence features of 11 polyphenol oxidases from plant and 5 from fungi, e.g. the two intracellular enzymes from *A. bisporus*, the shiitake mushroom *Lentinula edodes* and the filamentous fungi *N. crassa* and *Podospora anserina* [22,25]. This study led to the identification of the site of proteolytic processing within a linker region that is located between the core and the C-terminal domain of fungal polyphenol oxidases; it also predicted the presence of elements of secondary structure in the C-terminal domain [25].

In this study, we first aimed at producing a full-length form of tyrosinase 2 from *T. reesei* to investigate the proteolytic processing and for structure determination. Mutant variants of tyrosinase 2 were lacking the residues that are located in proximity of the cleavage site and that are possibly recognised by specific proteases. These mutant proteins were produced in *T. reesei* in a secreted form and, opposed to prediction, were still lacking the C-terminal domain. Suspecting an overall instability and sensitivity to proteolysis of the whole C-terminal domain, we analysed whether this domain possessed sequence features that could explain the phenomenon. Since tyrosinase 2 is the only secreted fungal tyrosinase characterised to date, we compared the features of its C-terminal domain to the ones of 22 similar predicted tyrosinases and nine well-characterised polyphenol oxidases described in literature. The presence of sequence features typical of unstable proteins, of intrinsically disordered regions and of proteolytic sites was considered.

2. Experimental

2.1. Cloning and overexpression of tyrosinase 2 mutants

The expression plasmid pMS190 carrying the gene for tyrosinase 2 between the cellobiohydrolase I (*cbh1*) promoter and terminator was used as template for the introduction of the desired mutations by overlap-extension PCR [32]. Two mutagenic oligonulceotides with opposite direction were designed for each desired mutation (Table 1). First, two overlapping mutated fragments were produced by PCR using a mutagenic oligonucleotide (Table 1) and an oligonucleotide annealing to either the *cbh1* promoter (cbh1-prom, and including a restriction site *SnaB*I site), or to the *cbh1* terminator (cbh1-term, and including a *Spe*I site). PCR reactions were carried out with the following conditions: 1 min of denaturation at 98 °C and 18 cycles 18 cycles of 98 °C for 1 min, annealing at 65 °C–1 °C per cycle for 50 s and extension at 72 °C for 1 min. In a second step, the fragments produced were isolated and combined. The PCR reaction included (i) 7 cycles of denaturation at 98 °C for 30 s, (ii) annealing at 65 °C–0.5 °C/cycle for 45 s and (iii) extension at 72 °C for 1 min 20 s. Oligonucleotides cbh1-prom and cbh1-term were then added and 18 additional cycles were carried out to produce the full-length mutated sequence. Amplification was performed with Phusion polymerase (New England Biolabs, Espoo, Finland). After double-digestion with *SnaB*I and *Spe*I, the fragments were cloned into pMS186 to give the corresponding expression plasmids (Table 1). Mutagenised plasmids were first transformed into *E. coli* DH5α and the presence of the desired mutation was checked by sequencing. Correct plasmids were transformed into the *T. reesei* strain VTT-D-00775 with a protoplast-based method

Table 1
Oligonucleotides and plasmids used in this study.

Mutant	Sequence in the protein[a]	Mutation	Plasmid	Sequence of oligos
TrTyr2	Y_{395}GPNSG↓KKRNAPR$_{407}$	–	pMS190	–
TrTyr2-NoCleav	Y_{395}GPNSGGSGSNAPS$_{407}$	K401G, K402S, R403G, R407S	pGF013	FW: ggg ccc aac tcg ggc ggc tcc ggc aac gcc ccg tcc gac ttc ttg agc; REV: gct caa gaa gtc gga cgg ggc gtt gcc gga gcc gcc cga gtt ggg ccc
TrTyr2-FactorXa	Y_{395}GPNSGGSGIEGR↓DF$_{409}$ (native R407 as cleavage site)	K401G, K402S, R403G, N404I, A405E, P406G	pGF014	FW: ggg ccc aac tcg ggc ggc tcc ggc atc gag ggc cgc gac ttc ttg agc; REV: gct caa gaa gtc gcg gcc ctc gat gcc gga gcc gcc cga gtt ggg ccc
TrTyr2-noLys	Y_{395}GPNSGGRRNAPR$_{407}$	K401G, K402R	pGF015	FW: ggg ccc aac tcg ggc ggc cgc cgc aac gcc ccg cgc gac ttc ttg agc; REV: gct caa gaa gtc gcg cgg ggc gtt gcg gcg gcc gcc cga gtt ggg ccc
TrTyr2-STOP	Y_{395}GPNSG$_{400}$	K401Stop	pGF016	FW: ggg ccc aac tcg ggc taa aag cgc aac gcc ccg cgc gac ttc ttg agc; REV:gct caa gaa gtc gcg cgg ggc gtt gcg ctt tta gcc cga gtt ggg ccc
TrTyr2-YG	A_{395}AGPNSGKKRNAPR$_{407}$	Y395A, G396A	pGF017	FW: cca gct ggc cgc ccc caa tcg gg; REV: ccc gag ttg ggg gcg gcc agc tgg

[a] The cleavage site in *T. reesei* is indicated by an arrow.