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Pichia pastoris production of a prolyl 4-hydroxylase derived from *Chondrosia reniformis* sponge: A new biotechnological tool for the recombinant production of marine collagen

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ABSTRACT

Prolyl 4-hydroxylase (P4H) is a $\alpha_2\beta_2$ tetramer catalyzing the post-translational hydroxylation of prolines in collagen. Its recombinant production is mainly pursued to realize biotechnological tools able to generate animal contaminant-free hydroxylated collagen. One promising candidate for biomedical applications is the collagen extracted from the marine sponge *Chondrosia reniformis*, because of its biocompatibility and because is devoid of the health risks associated with bovine and porcine collagens.

Here we report on the production and selection, by enzymatic and biomolecular analyses, of a triple transformed *Pichia pastoris* strain expressing a stable P4H tetramer derived from *C. reniformis* sponge and a hydroxylated non fibrillar procollagen polypeptide from the same animal. The percentage of recombinant procollagen hydroxylated prolines inside the transformed yeast was of 36.3% analyzed by mass spectrometry indicating that the recombinant enzyme is active on its natural substrate inside the yeast cell host. Furthermore, the recombinant sponge P4H has the ability to hydroxylate its natural substrate in both X and Y positions in the Xaa-Yaa-Gly collagenous triplets.

In conclusion this *Pichia* system seems ideal for high-level production of hydroxylated sponge- or marine-derived collagen polypeptides as well as of conotoxins or other marine proteins of high pharmacological interest needing this particular post-translational modification.

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1. Introduction

Q2 Prolyl 4-hydroxylase (P4H) is a key enzyme of the collagen biosynthesis catalyzing the hydroxylation of proline residues in -Xaa-Pro-Gly-sequences typical of proteins with collagen domains (for review, see Kivirikko et al., 1989; Kivirikko and Myllyharju, 1998; Myllyharju, 2003). The synthesis of 4-hydroxyproline residues is essential to the folding of the newly synthetized chains into the triple helices of collagens. In vertebrates, this enzyme is a $\alpha_2\beta_2$ tetramer, where the α subunits contain the catalytic active site for the proline hydroxylation, while the β subunits, that are identical to protein disulfide-isomerase (PDI), act to maintain the α subunits in a soluble form and to retain the enzyme in the ER lumen (Kivirikko et al., 1989). The pivotal role of this enzyme in collagen metabolism makes it a potential specific target for pharmacological regulation of fibrotic diseases and, for this reason, several studies have been carried out in order to elucidate the details of the hydroxylation mechanism (Myllyharju and Kivirikko, 1997; Lamberg et al., 1995). Attempts to assemble an active P4H tetramer *in vitro* from its individual α and β subunits have been unsuccessful (Koivu and Myllylä, 1986; Nietfeld et al., 1981). Human P4H was produced in recombinant form in insect cells by baculovirus vectors (Vuori et al., 1992) and more recently in *E. coli* (Kersteen et al., 2004). The recombinant production of a stable P4H tetramer proves to be of great significance not only to perform accurate structure/function analyses but also to promote further studies on the enzymatic activity mechanism by site-specific mutagenesis. Last but not least, this protein is considered a fundamental biotechnological tool for the production of recombinant proteins needing prolyl-hydroxylation in their post-translational modifications as collagen.

Due to its several industrial applications, the recombinant production of collagen and all its derivatives as gelatins has been

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pursued for many years in different biological systems as mammalian cells (Toman et al., 1999), tobacco plants (Ruggiero et al., 2000), silkworms (Tomita et al., 2003) and yeast cells (Vuorela et al., 1997). Among these systems, the yeast model proved to be the best performing in terms of yield/cost ratio (Olsen et al., 2003). With a few exceptions (De Bruin et al., 2002), the most commonly used species for this application, *i.e. S. cerevisiae* and *P. pastoris*, are devoid of endogenous P4H activity. Through a complex system of yeast cell triple transformation with both α and β P4H and collagenous genes, Vuorela and collaborators obtained a fully hydroxylated human procollagen type III in the methanotrophic yeast *P. pastoris*. Furthermore, in the same system it was observed that co-expression of type III procollagen was also able to enhance the enzymatic activity of the recombinant P4H itself (Vuorela et al., 1997).

Marine sponges are organisms naturally rich in collagens whose features are peculiar if compared to their vertebrate counterparts (Garrone et al., 1975). Sponges are an interesting alternative to the classical bovine and porcine sources for the extraction of these molecules, and their exploitation is increasing with time due to the health risks arisen from the use of bovine collagen (*i.e.* bovine spongiform encephalopathy, Moon et al., 2014) as well as to ethical concerns for the use of porcine collagen (for Muslim religious). There are several fields of applications of collagen extracts derived from marine sponges related to biomedicine, drug preparation and cosmetics, as for example the use of marine spongin as a biomimetic scaffold for human osteoprogenitor cell attachment, growth and differentiation (Green et al., 2003). One promising candidate for applications in humans is the collagen extracted from the marine sponge *Chondrosia reniformis*, because of its significant biocompatibility with human skin (Swatschek et al., 2002). The use of collagen extracts from *C. reniformis* was also proposed in the form of nanoparticles as penetration enhancers for the transdermal delivery of 17 beta-estradiol-hemihydrate in hormone therapy (Nicklas et al., 2009a) and as enteric coating for gastro resistant delayed-release tablets (Nicklas et al., 2009b). It was also observed that *C. reniformis* collagen can be used as an organic template for silica polymerization (Ehrlich et al., 2010). Recently, a *C. reniformis* non fibrillar collagen type was described at the molecular level (Pozzolini et al., 2012) and the full length cDNA coding for the α and β subunits of *C. reniformis* P4H were cloned and characterized (Pozzolini et al., 2015). To date, all the cosmetic and pharmaceutical preparations that use sponge collagen extracts are not characterized at the molecular level. Thus, the optimization of a system for the recombinant production of marine sponge collagen would allow the possibility to take advantage of safe and well-defined molecular types and furthermore, to limit the expensive procedures of sample recovery and purification as well as to avoid environmental issues due to sea pauperization of marine sponges.

In the present work, we produced a sponge collagen-specific proline-hydroxylating yeast expression system. Such system contains an active *C. reniformis* P4H enzyme by co-expression of its α and β subunits in *Pichia pastoris*. In particular, using four different *Pichia* strains and two different expression vectors for the αP4H we obtained eight different αP4H$^+$ yeast strains. The strain with the best recombinant enzymatic activity was then selected and further transformed with a non fibrillar collagen gene from *C. reniformis* marine sponge in order to assess the recombinant P4H activity directly inside the yeast cells on their natural target protein by mass spectrometric analysis of proline-hydroxylated collagen-derived peptides.

2. Materials and methods

2.1. Construction of expression vectors

A cDNA coding for *C. reniformis* αP4H polypeptide (GenBank an JQ699291), extending from the translation initiation codon to the stop codon, flanked by *Eco*RI restriction site in 5′-end and *Kpn*I in 3′-end, was synthetized by PCR and directionally ligated in pPink-Low copy (Life technologies, Milan, Italy) or in pPink-High copy, generating pPink-HC\α or pPink-LC\α vectors, respectively.

A cDNA coding for *C. reniformis* βP4H (PDI) (GenBank an JQ699292) extending from the codon for the first amino acid after the signal peptide cleavage site (1–16) to the stop codon, flanked by *Pml*I restriction site in 5′-end and *Not*I in 3′-end, was synthetized by PCR and directionally ligated in αpPIC6B generating the αpPIC6B\PDI vector. Finally the expression vector pPICZ\ColCH was obtained by directional ligation of a PCR amplified cDNA of a *C. reniformis* non fibrillar collagen (GenBank an DQ874470) extending from the translation codon initiation to the stop codon, flanked by *Pml*I restriction site in 5′-end and *Not*I in 3′-end.

2.2. Transformation, growth and induction of P. pastoris

Four different *Pichia pastoris* strains supplied by the *Pichia Pink* Expression System kit (Life Technologies) were made competent using *Pichia Easy Comp*™ Kit (Life Technologies) and co-transformed using 5 μg of *Spe*I-linearized pPink-HC/α or pPink-LC/α and 5 μg of *Sac*I-linearized αpPIC6/PDI, incubated for four days at 30 °C on adenine dropout selective agar plates added with 0.3 mg/ml Blasticidin (Life Technologies). Using four different *P. pastoris* strains and two different pPink expression vectors, eight different recombinant yeast strains were obtained (Table 1).

A *Pichia pastoris* strain co-expressing prolyl 4-hydroxylase and a *C. reniformis* non-fibrillar collagen polypeptide, ColCH, was then generated by transforming the competent strain αβH4 with 10 μg of *Pme*I-linearized pPICZ/colCH and selected in YPD agar plates with 0.1 mg/ml Zeocin (Life Technologies). The selected clones were cultured as described in the Manual of the PichiaPink™ Expression System. Shaker flask cultures were grown in a buffered glycerol

Table 1
Pichia pink strain and expression vectors used to generate the different *Pichia* strains.

Strain	Transformed strain	Expression vectors	Expressed polypeptide	Selection
αβH1	*Pichia pink 1*	pPinkHCα;αpPIC6BPDI	P4H[a]/amPDI[b]	-adenine/blasticidin
αβH2	*Pichia pink 2*	pPinkHCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβH3	*Pichia pink 3*	pPinkHCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβH4	*Pichia pink 4*	pPinkHCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβL1	*Pichia pink 1*	pPinkLCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβL2	*Pichia pink 2*	pPinkLCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβL3	*Pichia pikK 3*	pPinkLCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
αβL4	*Pichia pink 4*	pPinkLCα;αpPIC6BPDI	P4H/amPDI	-adenine/blasticidin
ColCH4	*αβ*H4	pPICZ/ColCH	P4H/amPDI/ColCh[c]	-adenine/blasticidin/zeocin

Expression vectors and selection strategies used to generate the different recombinant Pichia strains.
[a] P4H, *C. reniformis* prolyl 4-hydroxylase.
[b] amPDI, *C. reniformis* protein disufide isomerase where the 16-amino acid signal peptide was replaced with the *S. cerevisiae* alpha mating factor (am).
[c] ColCH, *C. reniformis* non fibrillar collagen.

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