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ORIGINAL ARTICLE

An expression system for the efficient incorporation of an expanded set of tryptophan analogues

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Abstract Biosynthetic incorporation of tryptophan (Trp) analogues in recombinant proteins using an *E. coli* Trp auxotroph expression host is limited to analogues modified with a small substituent like a fluoro atom or a hydroxyl or amine group. We report here the efficient incorporation (>89 %) of chloro- and bromo atoms containing Trp analogues in alloproteins at high expression levels using a *Lactococcus lactis* Trp auxotroph strain. This result was only obtained after coexpression of the enzyme tryptophanyl-tRNA synthetase (TrpRS) of *L. lactis*, an enzyme believed to show a more relaxed substrate specificity than TrpRS from *E. coli*. Chloro- and bromo-Trps are attractive intrinsic phosphorescence probes as these Trp analogues are much less sensitive for quenchers in the medium, like oxygen, than Trp. Coexpression of TrpRS was also essential for the biosynthetic incorporation (94 %) of the Trp analogue 5,6 difluoroTrp. This makes our expression system ideally suited to generate a set of methyl- and fluoro-substituted Trp analogue-containing alloproteins in high yield for investigating the involvement of the Trp residue in cation-pi or pi–pi interactions. Taken together, the presented Trp auxotroph expression system features the most relaxed specificity for Trp analogue structures reported to date and gives a high alloprotein yield.

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Introduction

Incorporation of non-canonical amino acids is an attractive strategy for introducing novel chemical and physical properties in recombinant proteins. For global replacement of an amino acid for its analogue in a recombinant protein an amino acid auxotroph expression strain is used, and the expression of alloproteins, proteins containing an unnatural amino acid, is relatively simple. A high incorporation efficiency of the amino acid analogue is often achieved together with a high alloprotein expression level (Hendrickson et al. 1990; Ross et al. 1997; van Hest and Tirrell 1998; Budisa et al. 1999). Only amino acid analogues which are structurally close to the natural amino acids can be incorporated in this way, a consequence of the high substrate specificity of the aminoacyl-tRNA synthetase (aaRS) enzymes. AaRS catalyzes the activation of its cognate amino acid using ATP and its coupling to the corresponding tRNA, yielding aminoacyl-tRNA. In recent years, several developments to expand the set of non-canonical amino acids, which can be incorporated under in vivo conditions, have been reported. Successful strategies include rational design of the amino-acid-binding pocket of aaRS or coexpression of aaRS in the expression host (Datta et al. 2002; Kiick et al. 2000; Kim et al. 2004; Kwon et al. 2006).

To date, a limited number of substituted tryptophan (Trp) analogues have been biosynthetically incorporated in proteins, namely mono-substituted fluoroTrps, aminoTrps, and hydroxyTrp analogues (Ross et al. 1997; Wong and Eftink 1997; Broos et al. 2003; Lepthien et al. 2006). Incorporating

Trp analogues containing a bulkier substituent in proteins was not successful (Budisa 2004; Ross et al. 1997) except in one study where a designed orthogonal aaRS/tRNA pair was introduced in *E. coli*, allowing incorporating 6-chloroTrp (6ClW) and 6-bromoTrp (6BrW), but not 5-BromoTrp (5BrW), at an amber codon position in the gene of the target protein (Kwon and Tirrell 2007). This is an attractive strategy to label proteins (Wang et al. 2001); however, development of an orthogonal aaRS/tRNA pair is not trivial and typically a low alloprotein yield is obtained.

Recently, we reported the use of a *L. lactis* Trp auxotroph as host to incorporate Trp analogues, including 5MeTrp (5MeW) (El Khattabi et al. 2008), a Trp analogue not translated by *E. coli* (Budisa et al. 2004). This result suggests that the substrate specificity of *L. lactis* tryptophanyl-tRNA synthetase (lacTrpRS) is more relaxed than *E. coli* tryptophanyl-tRNA synthetase (ecoTrpRS).

In this report, we present an approach to increase the number of Trp analogues which can be incorporated efficiently by *L.* lactis (Fig. 1). To achieve this, lacTrpRS was co-expressed together with the recombinant target protein. We show here that coexpression of lacTrpRS in the *L. lactis* Trp auxotroph results in efficient incorporation (89–98 %) of all investigated Trp analogues in recombinant proteins together with high expression levels.

Experimental

General DNA techniques and transformation of *L. lactis*

Standard recombinant DNA techniques were performed as described previously (Sambrook et al. 1989) or as specified by the manufacturers. Enzymes and buffers were purchased from BioLabs—-New England or Roche. Nucleotide sequence analysis and DNA synthesis were performed by BaseClear (Leiden, The Netherlands).

The *trpRS* gene encoding tryptophanyl-tRNA synthetase was amplified from *L. lactis* MG1363 chromosomal DNA via PCR using pFU Taq DNA polymerase and the following primers: trpRS_fw (5′-AAAGAGCTCAAAGGAGAAAAAAATGACAAAACC) and trpRS_rev (5′-AAACTGCAGAGGTGTCAAAACAATGAATTACC) containing *Sac*I and *Pst*I restriction sites, respectively. The amplified fragment was purified using a PCR purification kit (Qiagen) and subsequently cloned into the pMG36e expression vector (van de Guchte et al. 1989), using *Sac*I and *Pst*I restriction enzymes and T4 DNA ligase, yielding plasmid pMG36e-trpRS.

Plasmid pPA295, containing the W20LysM tandem gene (Petrovic et al. 2012) was derived from a nisin-inducible expression-secretion vector pNZ8048 (Kuipers et al. 1997) and electrotransformed into freshly prepared electrocompetent *L. lactis* PA1002 cells harboring the pMG36e-trpRS plasmid using a Bio-Rad Gene Pulser (Bio-Rad) (Holo and Nes 1995).

Chemo-enzymatic synthesis and purification of tryptophan analogues

5-hydroxytryptophan (5HW), 5-methyltryptophan (5MeW), and 5-BrTrp were from Sigma–Aldrich. 5-fluoroTrp (5FW), 5,6-difluoroTrp (5,6diFW), 6ClW, and 6BrlW were synthesized using a modified published procedure (Blaser et al. 2008). In brief, 17.5 mol acetic acid, 1.8 mmol acetic anhydride, 0.4 mmol of L-serine (Acros Organics) and

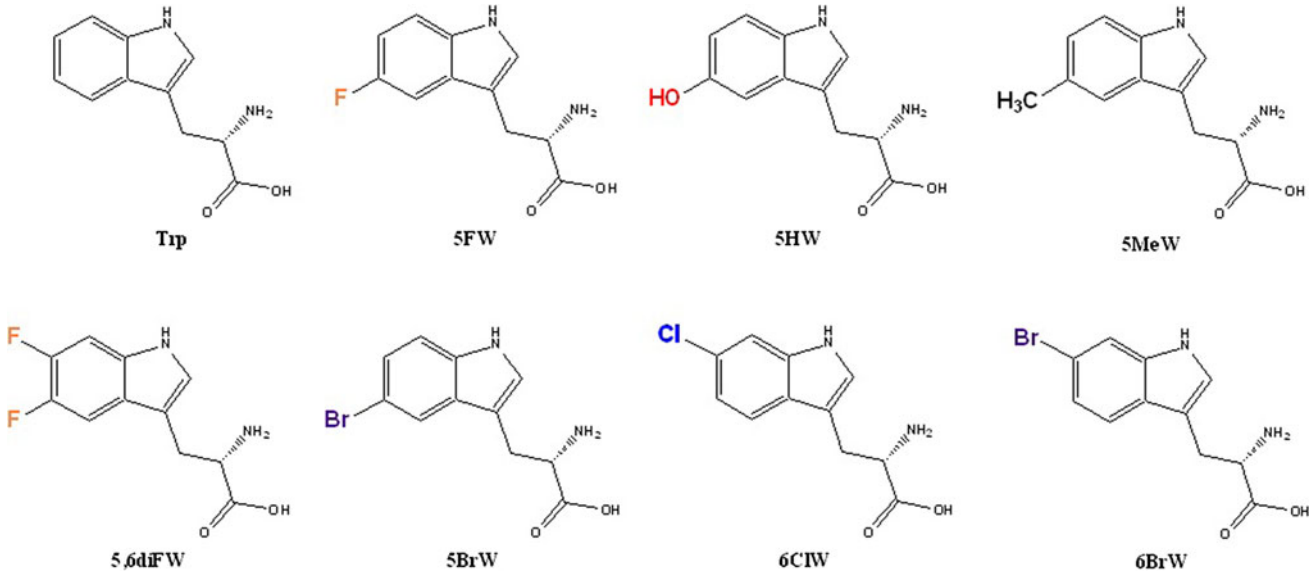

Fig. 1 Structures of tryptophan and its analogues used in this study: *Trp* tryptophan, *5FW* 5-fluorotryptophan, *5HW* 5-hydroxytryptophan, *5MeW* 5-methyltryptophan, *5,6diFW* 5,6-difluorotryptophan, *5BrW* 5-bromotryptophan, *6ClW* 6-chlorotryptophan, *6BrW* 6-bromotryptophan

0.2 mmol of indole analogue, 5F-indole (Sigma–Aldrich), 5,6diF-indole (Biosynth), 6Cl-indole (Biosynth), or 6Br-indole (Biosynth), respectively, were mixed and the solution deaerated via purging with argon gas. This solution was incubated for 3 h at 73 °C. Subsequently, 10 ml of 50 mM sodium-borate buffer, pH 8.0, containing 0.125 mM cobalt(II)-chloride was added to the reaction mixture, and the pH was adjusted to 8.0 with NaOH. Amano-acylase from *Aspergillus niger* (Sigma-Aldrich) was added at a concentration of 10 mg/ml, and the solution incubated for 48 h at 37 °C with shaking (250 rpm). The solution was centrifuged for 10 min at 5,400×*g* and the supernatant was loaded in 2.5 ml portions on a PD-10 desalting column (Amersham Bioscience), pre-equilibrated with 10 mM NaOH. Tryptophan analogues were eluted in 2 ml fractions from the column using 10 mM NaOH. Fractions with the highest A_{280}/A_{405} ratio were pooled and concentrated eight times in a vacuum concentrator (Eppendorf).

The purity of the synthesized Trp analogues was determined via silica-gel thin-layer chromatography (TLC) with butanol:H_2O:acetic acid (4:1:1) as mobile phase. Visualization was done in iodine vapor.

L. lactis growth, protein expression, and purification

Lactococcus lactis Trp auxotroph PA1002 (El Khattabi et al. 2008), harboring the pPA295 and pMG36e-trpRS plasmids, was grown overnight at 30 °C in GM17 broth [M17 (Oxoid) supplemented with 0.5 % (w/v) glucose] (Terzaghi and Sandine 1975), 5 μg/ml of chloramphenicol, and 75 μg/ml of erythromycin. Subsequently, 50 ml fresh GM17 medium supplemented with 5 μg/ml of chloramphenicol and 75 μg/ml of erythromycin was inoculated with this overnight culture (1:50) and growth at 30 °C was continued until an OD_{600} of 0.8. The cells were harvested by centrifugation at 5,400×*g* for 10 min at 30 °C, washed three times with PBS at 30 °C, and resuspended in 50 ml CDM (chemically defined medium) (Poolman and Konings 1988) supplemented with all amino acids, except Trp. After a starvation period of 30 min at 30 °C, nisin, 1 mM Trp or Trp analogue was added, as described (El Khattabi et al. 2008). The cells were subsequently incubated for 18 h at 30 °C.

The LysM tandem protein is secreted by *L. lactis* and it is the most abundant protein in the culture supernatant. After centrifugation, the supernatant was loaded on an Amicon concentrator device (MWCO 3 kDa) and washed with phosphate saline buffer (PBS), pH 7.4. LysM alloprotein concentrations were determined by comparing the intensity of the alloprotein bands with BSA as standard using a 12.5 % SDS-PAA gel stained with 0.05 % Coomassie Brilliant Blue R-250 solution, using GelQuant.NET software provided by Biochemlabsolutions.

Mass spectrometry

20 μl aliquots of W20LysM tandem protein (~0.1 mg/ml) in phosphate-buffered saline (PBS), pH 7.4 was exchanged to 100 mM ammonium bicarbonate buffer using an Amicon concentrator (molecular weight cut off 3 kDa). 2 μl of a porcine trypsin (Promega) stock solution (55 μg/ml) was added and the sample incubated for 3 h at 37 °C. 0.75 μl was spotted on a MALDI (matrix-assisted laser-desorption ionization) target and mixed immediately with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamate (LaserBio Labs) in 50 % acetonitrile/0.1 % (v/v) trifluoroacetic acid. Spots were recorded on a Voyager DE-PRO MALDI-TOF (time of flight) instrument (Applied Biosystems). Spectra were calibrated externally with standard peptides. Noise filter (correlation factor of 0.9) and Gaussian smoothing (filter width of 19 points) were applied to all spectra using Data Explorer (TM) software (Applied Biosystems). The Trp analogue incorporation efficiency was calculated by measuring the peak areas of the Trp analogue containing peptide and the Trp containing peptide.

Fluorescence

Steady-state fluorescence spectra were recorded with a Fluorolog-3.2.2 fluorimeter (Jobin–Yvon, Edison, NJ, USA). A protein concentration of approximately 0.1 mg/ml was used. W20LysM tandem containing 5FW, 5HW, and 5MeW, were excited at both 295 and 305 nm. Excitation slits were set up at 0.5 nm, emission slits were set at 5.00 nm, and 3 × 3 mm quartz cuvettes were used. Fluorescence emission was measured from 320 to 450 nm at 23 °C. Spectra were corrected for the PBS buffer emission.

Results

Cloning and overexpression of lacTrpRS

The sequence of the complete genome of the lactic bacterium *L. lactis* subsp. *cremoris* MG1363 is known (NCBI, GenBank: AM406671.1 http://www.ncbi.nlm.nih.gov/nuccore/AM406671) (Wegmann et al. 2007) and we identified the *trpRS* gene in the locus llmg_0079. Using PCR, a fragment of 1,075 base pairs (bp) comprising the *trpRS* nucleotide sequence (1,025 bp) and a putative ribosome-binding site was amplified and cloned into the pMG36e plasmid (van de Guchte et al. 1989) behind the strong constitutive promoter P32 (van der Vossen et al. 1987). The newly constructed plasmid, pMG36e-trpRS, was transformed into the *L. lactis* PA1002 Trp auxotroph strain (El Khattabi et al. 2008).

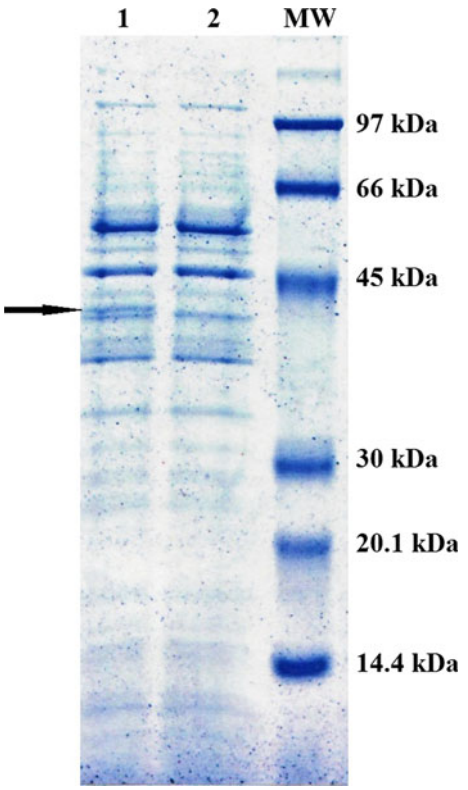

Fig. 2 Expression of lacTrpRS recombinant protein in *L. lactis* PA1002, visualized via SDS-PAGE. *Lane 1*. Whole cell extracts of *L. lactis* PA1002 harboring the lacTrpRS encoding pMG36e-trpRS plasmid (*black arrow* indicate lacTrpRS band); *Lane 2 L. lactis* PA1002 harboring empty pMG36e plasmid. Cells were harvested at $OD_{600} \sim 0.7$. MW is the molecular mass standard

SDS-PAGE analysis of these cells harvested at $OD_{600} \sim 0.7$ revealed the presence of a band at approximately 38 kDa (theoretical mass of lacTrpRS is 37,963 Da). This band was not visible in a control experiment (Fig. 2). This experiment demonstrated that lacTrpRS enzyme is well overexpressed in *L. lactis*, before the induction of the target protein expression is initiated.

Biosynthetic incorporation of Trp analogues into the W20 LysM tandem protein

The *L. lactis* Trp auxotroph strain PA1002, harboring plasmid pMG36e-trpRS, was transformed with the plasmid encoding the single Trp containing target protein, W20LysM tandem (Petrovic et al. 2012). The W20LysM tandem and the lacTrpRS containing plasmids comprise similar type of replication origins, pWV01 (van de Guchte et al. 1989) and pSH71 (de Ruyter et al. 1996), respectively, which are compatible and contain different selection markers, chloramphenicol, and erythromycin, respectively. Expression of the W20LysM tandem protein was performed in CDM supplemented with 1 mM Trp or Trp analogue. Excellent expression levels of W20LysM tandem alloproteins were observed in all cultures (25–50 mg per 1 L of culture). Furthermore, due to the method used, the target LysM protein was the only protein detectable in the culture supernatant (Fig. 3), facilitating its subsequent characterization. Only in the presence of 5BrW or 6BrW slightly lower expression levels were observed (Fig. 3). The high yield of alloproteins containing 5HW, 5,6diFW, 6ClW, 5BrW, and 6BrW is due to the coexpression of lacTrpRS. When *L. lactis* PA1002 comprises only the W20LysM tandem-containing plasmid, a very low or no detectable expression of W20LysM containing these Trp analogues was observed (Fig. 4). The 5FW- and 5MeW-containing W20LysM tandem expression levels were found only minimally dependent on coexpression of lacTrpRS (data not shown).

The incorporation efficiency of each Trp analogue in the target protein was established by MALDI-TOF mass

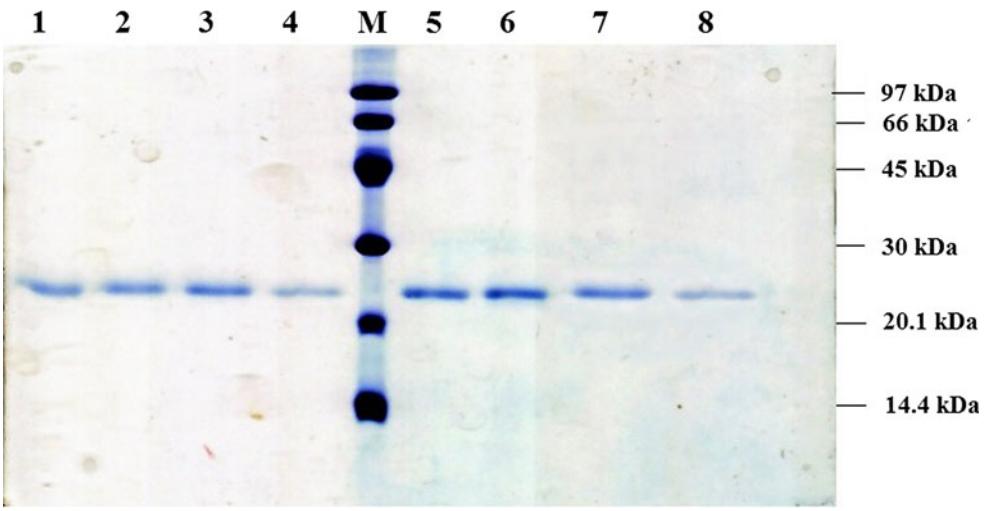

Fig. 3 SDS-PAGE of culture supernatants after overnight expression of W20LysM tandem in the presence of Trp or a Trp analogue. W20LysM tandem expressed in the presence of *Lane 1* Trp; *Lane 2* 5FW; *Lane 3* 5HW; *Lane 4* 5BrW; *Lane 5* 5MeW; *Lane 6* 5,6diFW; *Lane 7* 6ClW; *Lane 8* 6BrW. *M* is the molecular mass standard

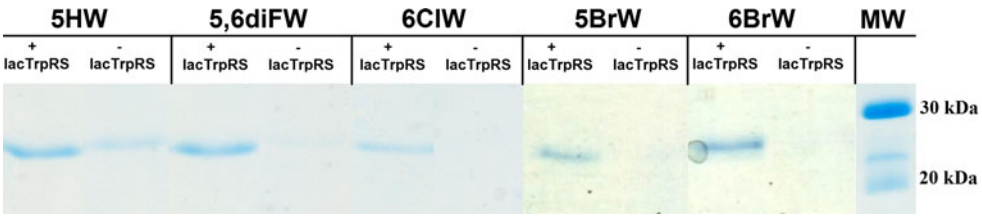

Fig. 4 SDS-PAGE of culture supernatants of *L. lactis* coexpressing lacTrpRS [+] or without coexpressing lacTrpRS [−]. Samples were collected after overnight expression of W20LysM tandem in the presence of 5HW, 5,6diFW, 6ClW, 5BrW, or 6BrW, respectively. MW is the molecular mass standard

spectrometry. For W20LysM tandem expressed in CDM supplemented with Trp, a tryptic peptide with a mass of 1,629.67 Da was detected (Fig. 5). This intense peak was absent or its intensity strongly reduced for W20LysM tandem expressed in the presence of a Trp analogue. In the mass spectra of these samples a new peak was found corresponding to the mass of the Trp analogue containing peptide (Fig. 5). Very high incorporation efficiencies (≥94 %) were found for 5FW, 5HW, 5MeW, and 5,6diFW, while for the other Trp analogues the incorporation efficiency was ∼90 % (Table 1).

W20LysM tandem alloproteins containing 5FW, 5HW, and 5MeW show a comparable fluorescence quantum yield (Q) as W20LysM tandem containing Trp. The identical shape of the emission spectra, when excited at 295 or 305 nm, supports the view that the presence of Trp in these

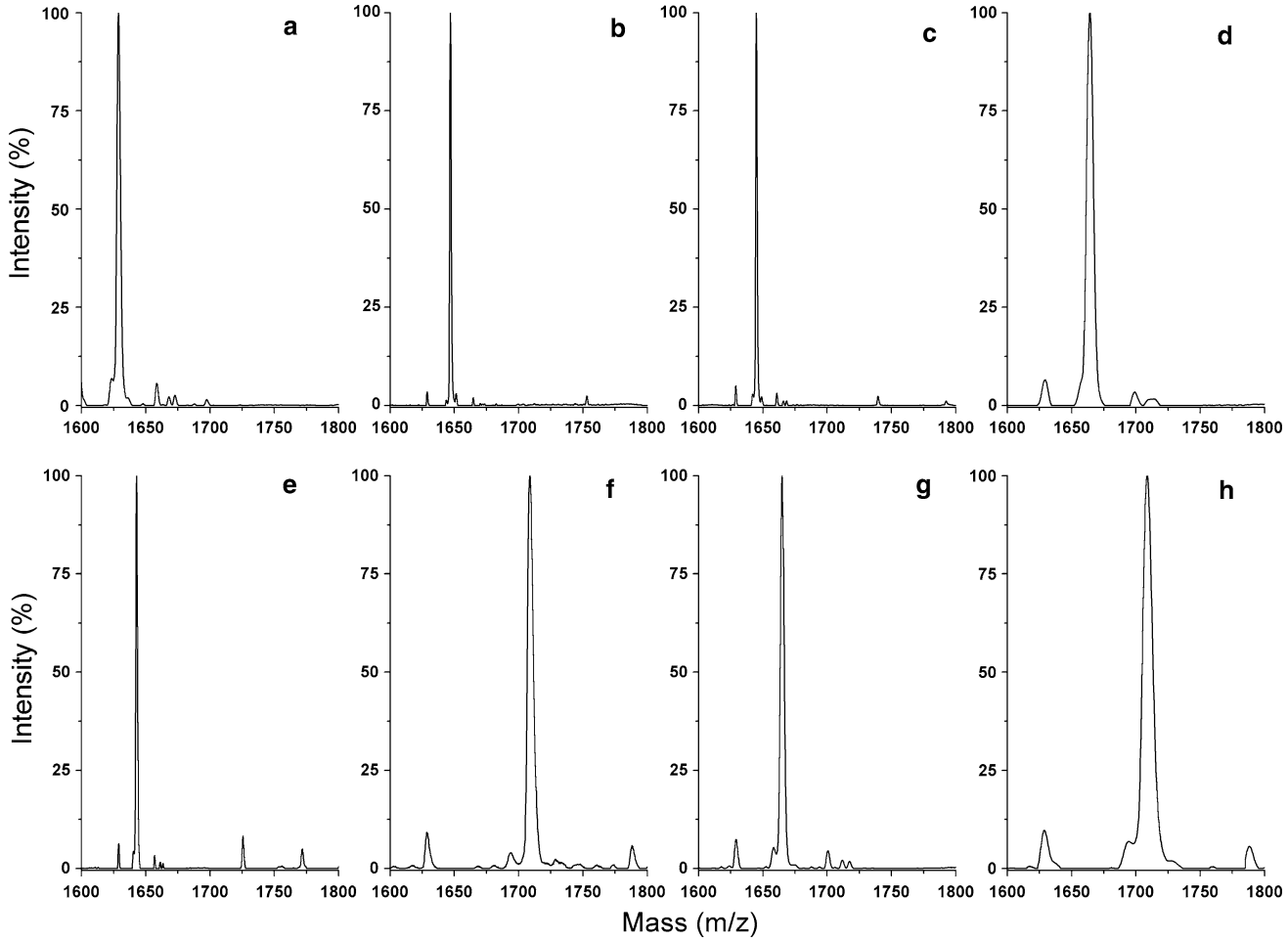

Fig. 5 MALDI-TOF spectra of tryptic peptide of W20LysM containing Trp or a Trp analogue. The mass region of the WGISVAQIQSANNLK peptide is shown. The peak of protonated peptide containing Trp (**a**), 5FW (**b**), 5HW (**c**), 5,6diFW (**d**), 5MeW (**e**), 5BrW (**f**), 6ClW (**g**), and 6BrW (**h**), respectively, are shown

Table 1 % Trp analogue incorporation in W20 LysM tandem protein

Trp analogue	Incorporation[a] (%)
5FW	98 ± 1
5HW	95 ± 1
5BrW	90 ± 2
5MeW	95 ± 1
5,6diFW	94 ± 2
6ClW	91 ± 2
6BrW	89 ± 3

[a] Data based on two independent experiments

three protein samples is very low (Fig. 6). The fluorescence Q of W20LysM tandem alloproteins containing 5,6diFW, 5BrW, 6ClW, and 6BrW, respectively, is very low, <0.01 (data not shown). Such a low fluorescence Q does not allow accurate comparison of emission spectra when excited at 295 and 305 nm wavelengths, respectively.

Discussion

In this work we report an efficient strategy to incorporate Trp analogues including those not biosynthetically introduced in proteins before, with high incorporation efficiency and resulting in a high alloprotein yields. The single Trp containing protein, W20LysM tandem was used as target protein for the incorporation of Trp analogues. W20LysM tandem is a small 20 kDa protein and a 3D model of this protein suggests the Trp residue is at a surface exposed position (Petrovic et al. 2012). By choosing a surface-exposed Trp position instead of a buried position minimizes the chance that the incorporation of a bulky Trp analogue (e.g. 5BrW and 6BrW) compromises folding and stability of the alloprotein.

Initial studies with 5BrW using the *L. lactis* Trp auxotroph PA1002 as expression host hinted that this analogue can be incorporated by *L. lactis*, as a very low amount of alloprotein was obtained. Cloning of the lacTrpRS behind the constitutive P32 promoter and its coexpression together with the target protein resulted in large increases in the expression levels of alloproteins containing 5,6diFW, 6ClW, 5BrW, and 6BrW, respectively. Expression levels become approximately equal as with Trp in the medium (~50 mg/L) only for the alloproteins containing 5BrW and 6BrW expression levels were ~50 % lower. 5HW can also be incorporated without lacTrpRS coexpression; however, a lower alloprotein yield (Fig. 4), and a lower incorporation efficiency (89 versus 95 %) are observed (El Khattabi et al. 2008). Similarly, the 5MeW incorporation efficiency increases from 92 to 95 % when lacTrpRS was coexpressed.

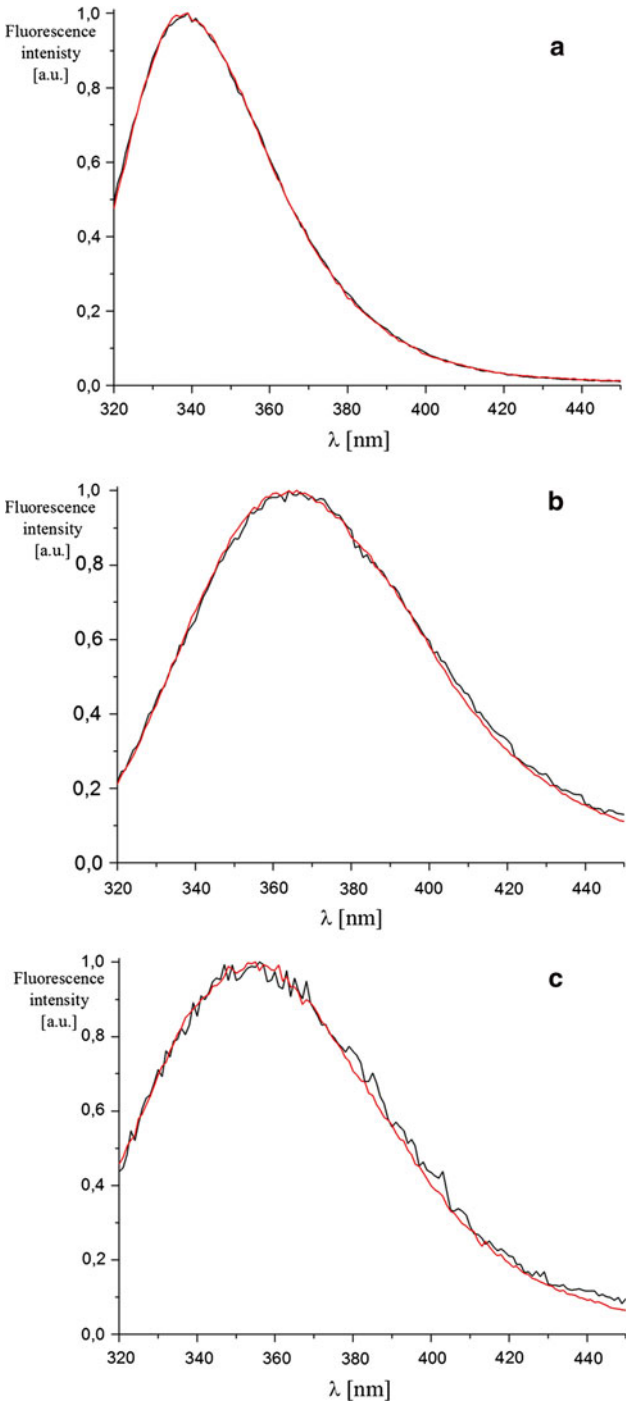

Fig. 6 Emission spectra of W20LysM tandem alloproteins excited at 295 and 305 nm, respectively. *Red line* emission spectrum of W20LysM tandem excited at 295 nm, *black line* emission spectrum of W20LysM tandem excited at 305 nm; **a** 5HW; **b** 5FW; **c** 5MeW containing W20LysM tandem. Spectra were normalized

The new alloproteins, which can now be produced relatively easily, offer novel opportunities in protein structure and function studies. For example, evaluation of a set of alloproteins containing a Trp with electron- donating

(methyl) or -withdrawing (fluoro) substituents in the indole moiety is a powerful means to study the role of a Trp residue in cation–pi or pi–pi interactions (Zhong et al. 1998). So far, alloproteins used in these studies were produced in very low amounts via an in vitro methodology using *Xenopus lavis* oocytes as expression system. The chloro- and bromo substituted Trp analogues offer interesting opportunities as intrinsic phosphorescence probes. Trp phosphorescence spectroscopy is one of the most sensitive methodologies known to probe local viscosity in a protein and is also ideally suited to investigate if the protein is present in different conformational states (Vanderkooi et al. 1987; Veldhuis et al. 2005). Widespread use of Trp phosphorescence is limited by the extreme sensitivity of the triplet state for quenchers, like oxygen (Strambini et al. 2004). For reliable measurements, the quencher concentrations in the buffer need to be reduced to the sub nM concentration range. This high sensitivity is a result of the very long intrinsic triplet lifetime (6.5 s) of Trp (Strambini et al. 2004), giving quenchers "ample time" to deactivate the triplet state. Chloro or bromo substitution of Trp strongly reduces the intrinsic triplet lifetime (Milton et al. 1978), making it 1–3 orders of magnitude less sensitive for quenchers like oxygen. Moreover, these substituents enhance the intersystem crossing rate from the singlet to the triplet state, thus suppressing fluorescence and increasing the phosphorescence intensity. We are currently investigating the spectroscopic properties of the various alloproteins via phosphorescence methodologies.

In summary, a versatile Trp analogue expression system was developed, allowing the efficient biosynthetic incorporation of Trp analogues not reported before together with a high alloprotein yield.

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