Biochimica et Biophysica Acta 1800 (2010) 986–992

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Bulky high-mannose-type N-glycan blocks the taste-modifying activity of miraculin

Keisuke Ito [a], Taishi Sugawara [a], Ayako Koizumi [a], Ken-ichiro Nakajima [a], Akiko Shimizu-Ibuka [a], Mitsunori Shiroishi [b], Hidetsugu Asada [b], Takami Yurugi-Kobayashi [b], Tatsuro Shimamura [b], Tomiko Asakura [a], Katsuyoshi Masuda [c], Masaji Ishiguro [c], Takumi Misaka [a], So Iwata [b,d,e], Takuya Kobayashi [b,d,*], Keiko Abe [a,*]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Iwata Human Receptor Crystallography project, ERATO, JST, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan
[c] Suntory Institute for Bioorganic Research, Wakayamadai 1-1-1, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan
[d] Department of Medical Chemistry, Kyoto University Faculty of Medicine, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan
[e] Division of Molecular Biosciences, Membrane Protein Crystallography Group, Imperial College London, London SW7 2AZ, UK

ARTICLE INFO

Article history:
Received 4 February 2010
Received in revised form 17 May 2010
Accepted 4 June 2010
Available online 11 June 2010

Keywords:
Miraculin
Sweet protein
Taste
Yeast expression system
N-glycan
Steric hindrance

ABSTRACT

Background: Miraculin (MCL) is a taste-modifying protein that converts sourness into sweetness. The molecular mechanism underlying the taste-modifying action of MCL is unknown.
Methods: Here, a yeast expression system for MCL was constructed to accelerate analysis of its structure–function relationships. The *Saccharomyces cerevisiae* expression system has advantages as a high-throughput analysis system, but compared to other hosts it is characterized by a relatively low level of recombinant protein expression. To alleviate this weakness, in this study we optimized the codon usage and signal-sequence as the first step. Recombinant MCL (rMCL) was expressed and purified, and the sensory taste was analyzed.
Results: As a result, a 2 mg/l yield of rMCL was successfully obtained. Although sensory taste evaluation showed that rMCL was flat in taste under all the pH conditions employed, taste-modifying activity similar to that of native MCL was recovered after deglycosylation. Mutagenetic analysis revealed that the N-glycan attached to Asn42 was bulky in rMCL.
Conclusions: The high-mannose-type N-glycan attached in yeast blocks the taste-modifying activity of rMCL.
General significance: The bulky N-glycan attached to Asn42 may cause steric hindrance in the interaction between active residues and the sweet taste receptor hT1R2/hT1R3.

1. Introduction

Miraculin (MCL) is a naturally occurring protein in the fruit of the West-African plant *Richadella dulcifica* [1]. It is a homodimer that consists of two glycosylated 191-amino acid polypeptides cross-linked by disulfide bonds [2–4]. MCL is of particular interest because it has unique taste-modifying properties. Though flat in taste at neutral pH, it shows taste-modifying activity and converts sourness to sweetness at acidic pH [5]. Although this interesting sensory effect has been characterized, the molecular mechanism underlying the taste-modifying action of MCL is unknown.

Although sweet proteins exhibit no structural similarity, the importance of charged Arg and Lys residues was confirmed by the mutagenetic studies [6–8]. MCL is a basic protein similar to other sweet proteins [2], but the role of the charged residues is not fully elucidated [5]. In the case of another taste-modifying protein, neoculin, its sweetness depends on a structural change due to the pH change, although the histidine residues may always playing a key role [9,10]. Our mutagenetic study of MCL using the *Aspergillus oryzae* expression system [11] showed that the histidine residue His30, located at the interface of the two monomeric MCL subunits, is one of the candidate active residues. As well as *A. oryzae*, other recombinant MCL (rMCL) expression systems have been reported previously [12,13]. To accelerate analysis of the structure–function relationships of the taste-modifying activity, however, it would be necessary to have a high-throughput expression system.

The yeast *Saccharomyces cerevisiae* expression system has advantages as a high-throughput analysis system [14,15], and we reported an advanced method useful for expressing proteins bearing various

Abbreviations: MCL, miraculin; rMCL, recombinant miraculin; nMCL, native miraculin; dgrMCL, deglycosylated recombinant miraculin

* Corresponding authors. Kobayashi is to be contacted at Laboratory of Cell Biology, 3rd Flr., Bldg. A, Graduate School of Medicine, Kyoto University, Yoshida-konoe-cho, Sakyo-ku, Kyoto, 606-8501, Japan. Tel.: +81 75 753 4386; fax: +81 75 753 4660. Abe, Laboratory of Biological Function, 3rd Flr., Bldg. 7B, Graduate School of Agricultural and Life Sciences, The University of Tokyo 1-1-1, Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan. Tel.: +81 3 5841 5129; fax: +81 3 5841 8006.
E-mail addresses: t-coba@mfour.med.kyoto-u.ac.jp (T. Kobayashi), aka7308@mail.ecc.u-tokyo.ac.jp (K. Abe).

0304-4165/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2010.06.003

mutations [16]. Using this advanced method, mutated proteins can be expressed within five days of mutation design. In addition, yeast can secrete folded proteins like *A. oryzae*. These characteristics of yeast expression systems render them suitable for high-throughput mutagenetic analysis. *S. cerevisiae*, however, has the disadvantage of relatively low expression levels of recombinant protein compared to other hosts [17]. To alleviate this weakness, in this study we focused on codon usage and signal-sequence as the first step of the system. These factors play an important role in high-level expression of secretory protein. Finally, through optimization of these factors, a 2 mg/l yield of rMCL in media was successfully obtained. As the second step, the yeast rMCL was purified and its taste-modifying activity was analyzed. Although taste-modifying activity was not initially detected in the purified rMCL, it was significantly recovered by deglycosylation treatment. The structural effects of glycosylation on the taste-modifying activity are discussed, along with the results of the mutagenetic study.

2. Materials and methods

2.1. Codon optimization

Codon-optimized genes were designed based on the protein sequence of MCL (GenBank Accession No. BAA07603) according to the codon bias of *S. cerevisiae* [18] (http://www.kazusa.or.jp/codon). The genes were synthesized by Takara (Otsu, Japan). Codon adaptation index (CAI) values were estimated using the JCat program [19]. CAI is a measure of directional synonymous codon usage bias defined in reference [20], and values clearly parallel the levels of gene expression. The index uses a reference set of highly expressed genes from a species to assess the relative merits of each codon, and a score for a gene is calculated from the frequency of the use of all the codons in that gene.

2.2. Yeast transformation

The gene fragments of signal-sequences (codon-optimized) were synthesized by primer dimerization using various primers (Fig. S1). To obtain the PCR fragments of MCL, gene-specific primers were used as follows: 5′-*GGTGGTGGTGATTATAAAGATGATGATGATAAA***GATTCGGCACCCAATCCGGTTCTTG**-3′ and 5′-*AAATTGACCTTGAAAATATAAATTTTCCCC***TCATTAGAAGTATACGGTTTTGTTGAAC**-3′ for native-codon MCL, and 5′-*GGTGGTGGTGATTATAAAGATGATGATGATAAA***GATTCTGCTCCAAATCCAGTTTTGGACATT**-3′ and 5′-*AAATTGACCTTGAAAATATAAATTTTCCCCC*-**TATTAGAAGTAAACAGTTTTTTGG**-3′ for codon-optimized MCL. Primers contained a gene-specific region (bold) and a homologous region (*italic*). PCR fragments, signal-sequence and the *Sma*I-linearized pRS426 GAL1 vectors were co-transformed into *S. cerevisiae* strain FGY217 [21]. Transformation was performed as described previously [16].

2.3. Liquid expression

For expression-screening of rMCL, transformants were selected and grown in −Ura selection medium (0.2% yeast synthetic drop-out medium without Ura, 0.7% yeast nitrogen base without amino acids and 2% glucose). After growing at 30 °C for 24 h, cells were harvested by centrifugation and resuspended in an equal volume of expression medium (1% casamino acids, 1.5% yeast nitrogen base without amino acids and 2% galactose, pH 4.0) at a final OD_{600} of 7 and grown at 20 °C for 24 h. The culture supernatant was collected and analyzed using anti-MCL antibody [11]. The expression yields were estimated by the detection level in comparison with that of native MCL (nMCL). For over-expression conditions, after growing in −Ura selection medium at 30 °C for 24 h, cells were transferred into growth medium (1% peptone, 0.5% yeast extract and 2% glucose) and grown at 30 °C for 24 h. Cells were harvested by centrifugation, resuspended in a half-volume of expression medium at a final OD_{600} of 14, and grown at 20 °C for 24 h.

2.4. On-plate detection of secreted MCL

The signal-sequence optimization method is illustrated in Fig. 1. Twelve yeast signal-sequences selected according to the report by Sahara et al. [22] and MCL signal-sequence were synthesized by primer dimerization (Fig. S1). After co-transformation with a mixture of 13 signal-sequences and MCL PCR fragment, cells were distributed on a −Ura expression plate (2% agarose, 0.2% yeast synthetic drop-out medium without Ura, 0.7% yeast nitrogen base without amino acids, 0.1% glucose, 2% galactose) and covered by a PVDF membrane. Colonies were grown at 30 °C for 24 h and 20 °C for 72 h. The membrane was analyzed by immunodetection using anti-FLAG antibody.

2.5. Purification and deglycosylation

rMCL was purified by hydrophobic, cation-exchange and size-exclusion column chromatographies [11]. Purity and yield of purified rMCL were evaluated by SDS-PAGE. For deglycosylation, endoglycosidase F1 was added to purified rMCL. After incubation at 30 °C for 12 h [23], the enzyme was removed by size-exclusion chromatography.

2.6. Sensory analysis of the taste-modifying activity

The taste-modifying activity of MCL was evaluated by a panel of well-trained members. In the preliminary experiment, 0.5 mg/ml rMCL was equivalent to 0.1 mg/ml nMCL in terms of taste-modifying activity at pH 3.0, and the specific activity of rMCL was estimated to be about 0.2-fold that in the nMCL. Thus, 100 μl of MCL solution, 0.1 mg/ml for nMCL or 0.5 mg/ml for rMCL, was applied to the tongue and held for 1 min, and the mouth was then rinsed with water. Then 400 μl of 100 mM sodium citrate buffer at pH 7.0, 5.0 or 3.0 was applied to the tongue and the sweetness was evaluated by comparing the taste of these solutions with those of standard aspartame solutions. Sweetness scores were represented according to the concentration of aspartame: score 9, >2 mM; score 8, 2 mM; score 7, 1–2 mM; score 6, 1 mM; score 5, 0.5–1 mM; score 4, 0.5 mM; score 3, 0.25–0.5 mM; score 2, 0.25 mM; and score 1, <0.25 mM.

2.7. Mutation analysis at the glycosylation sites

The site-directed mutagenesis method using *S. cerevisiae* was described previously [16]. To mutations at positions 42 or 186, the following gene-specific primers were used: 5′-CTGTTTCTGCTACTACTCCACAAGGTACTTTCGTTTGTCCACCTAGAGTTGTCCAAAC-3′ and 5′-GTTTGGACAACTCTAGGTGGACAAACGAAAGTACCTTGTGGAGTAGTAGCAGAAACAG-3′ for N42Q, 5′-GATAAACCATTCGCTTTCGAGTTCCAAAAAACTGTTTACTTCTAATAGGGGGAAAATTTATATTTTCAAGGTCAATTT-3′ and 5′-AAATTGACCTTGAAAATATAAATTTTCCCCCTATTAGAAGTAAACAGTTTTTTGGAACTCGAAAGCGAATGGTTTATC-3′ for N186Q. Primers contained a mutation site (underlined). PCR fragments, SSP120 signal-sequence and the *Sma*I-linearized pRS426 GAL1 vectors were co-transformed into *S. cerevisiae* strain FGY217 [21]. Mutated proteins were expressed under screening conditions (see Section 2.3), and the culture supernatant was probed by immunoblotting analysis using anti-MCL antibody [11].

3. Results

3.1. Enhanced expression of miraculin through codon optimization

To improve the expression level, MCL codon usage was optimized for yeast codon frequencies (see Section 2). Fig. 2 shows the gene sequence of native and optimized MCL. rMCL was detected as a broad