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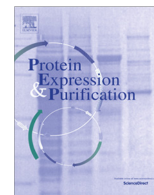

High-level soluble expression of a bacterial N-acyl-D-glucosamine 2-epimerase in recombinant *Escherichia coli*

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ABSTRACT

N-Acyl-D-glucosamine 2-epimerase (AGE) is an important enzyme for the biocatalytic synthesis of *N*-acetylneuraminic acid (Neu5Ac). Due to the wide range of biological applications of Neu5Ac and its derivatives, there has been great interest in its large-scale synthesis. Thus, suitable strategies for achieving high-level production of soluble AGE are needed. Several AGEs from various organisms have been recombinantly expressed in *Escherichia coli*. However, the soluble expression level was consistently low with an excessive formation of inclusion bodies. In this study, the effects of different solubility-enhancement tags, expression temperatures, chaperones and host strains on the soluble expression of the AGE from the freshwater cyanobacterium *Anabaena variabilis* ATCC 29413 (AvaAGE) were examined. The optimum combination of tag, expression temperature, co-expression of chaperones and host strain (His_6-tag, 37 °C, GroEL/GroES, *E. coli* BL21(DE3)) led to a 264-fold improvement of the volumetric epimerase activity, a measure of the soluble expression, compared to the starting conditions (His_6-MBP-tag, 20 °C, without chaperones, *E. coli* BL21(DE3)). A maximum yield of 22.5 mg isolated AvaAGE per liter shake flask culture was obtained.

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Introduction

The 9-carbon monosaccharide *N*-acetylneuraminic acid (Neu5Ac) is the most abundant member of the family of sialic acids [1]. These amino sugars are mainly found at the non-reducing end of glycoconjugates and play a role in a multitude of cellular processes. Some physiological roles are linked to their negative charge, such as the transportation of ions and the stabilization of proteins [2]. When presented on cellular membranes, at the end of oligosaccharide chains of glycosylated lipids and membrane proteins, sialic acids play an important part in cell recognition and communication, modulation of cell receptors, and tumor metastasis [2,3]. Dietary sialic acids have been found to be essential nutrients for the development of the human brain and its cognitive function [4]. Sialylated molecules are also the targets for binding by many pathogens and represent specific markers for some types of cancer [2,3,5]. For these reasons, Neu5Ac and its derivatives are of importance for the development and production of dietary supplements and various pharmaceuticals [4,6,7]. A well-known example is the compound 4-guanidino-Neu5Ac2en (Zanamivir), which is a potent inhibitor of neuraminidases produced by influenza A and B viruses [7].

Neu5Ac can be obtained by several methods such as the extraction from natural sources [8,9], hydrolysis of colominic acid (a natural Neu5Ac homopolymer) [10], organic synthesis [11,12], chemoenzymatic catalysis [13,14], and biocatalysis employing isolated enzymes [15–17] or engineered whole cells [18–23]. However, the demand for Neu5Ac has greatly exceeded its availability from natural sources and its organic synthesis involves complex protecting group chemistry. In recent years, particular attention has been given to diverse biocatalytic routes. These have a great potential to be more cost-effective and environmentally sustainable than the chemoenzymatic method, which is currently used for the major part of industrial Neu5Ac production [6,24,25]. However, a completely biocatalytic process has also been already industrialized [25].

The majority of biocatalytic approaches for Neu5Ac synthesis involve an *N*-acylglucosamine 2-epimerase (AGE, EC 5.3.1.8) in combination with an *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3) [15–21]. The AGE[1] epimerizes the cheap substrate *N*-acetyl-D-glucosamine (GlcNAc) to *N*-acetyl-D-mannosamine (ManNAc), which

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[1] *Abbreviations used:* AGE, *N*-acyl-D-glucosamine 2-epimerase; AvaAGE, *N*-acyl-D-glucosamine 2-epimerase from *Anabaena variabilis* ATCC 29413; GlcNAc, *N*-acetyl-D-glucosamine; GST, glutathione-S-transferase; Hsp, heat shock protein; ManNAc, *N*-acetyl-D-mannosamine; MBP, maltose-binding protein; NAL, *N*-acetylneuraminate lyase; Neu5Ac, *N*-acetylneuraminic acid; pAGE, *N*-acyl-D-glucosamine 2-epimerase from porcine kidney; SBP, streptavidin-binding peptide; SET, solubility-enhancement tag; SUMO, small ubiquitin-like modifier; TEV, tobacco etch virus; Tf, trigger factor.

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is more expensive and not readily available. In a second step, ManNAc undergoes a NAL-catalyzed aldol condensation with pyruvate to form Neu5Ac. Neu5Ac production using whole cells faces several challenges such as mass transfer limitations by the cell membrane and the occurrence of competing side reactions. Much work has been done during the last years to overcome these problems, because although the isolated enzyme process is regarded as efficient, high costs arise mainly from (1) the usually strong dependence of AGEs on allosteric activation by ATP and (2) the need for (partial) protein purification [19–21]. The isolation of AGEs is further complicated by the extensive formation of inclusion bodies upon recombinant expression in *Escherichia coli*, which is a shared characteristic of all currently known AGEs [18,26–29].

The identification of an AGE from the freshwater cyanobacterium *Anabaena* ATCC 29413 (AvaAGE) with high specific activity (117.0 ± 1.6 U mg^{-1} at 37 °C) and low dependence on allosteric activation eliminated the first disadvantage of the isolated enzyme process, i.e., the need for ATP addition [28]. Without allosteric activation by nucleotides, AvaAGE reached up to 32% of the activity measured with ATP in excess. As a result, the maximum specific activity of AvaAGE in the absence of ATP (37.7 ± 0.9 U mg^{-1}) was 16% higher than the activity of the porcine AGE, which has been most commonly used in preparative applications [16,25], at 1 mM ATP (32.5 ± 2.7 U mg^{-1}) [28,30].

The aim of this study was to address the second major drawback of the isolated enzyme process, i.e., to minimize the costs for protein purification by maximizing the yield of soluble AGE obtained by recombinant expression in *E. coli*. Various strategies have been developed to improve the soluble expression of recombinant proteins in *E. coli* [31,32]. One possible route is to use solubility-enhancing fusion tags such as maltose-binding protein (MBP) or glutathione-S-transferase (GST). Other strategies avoid the modification of the target protein and focus on the expression conditions, the host strain or the co-expression of molecular chaperones. In this study, the effects of seven solubility-promoting tags, varying expression temperatures and co-expression of chaperones in different host strains on the soluble expression of AvaAGE were examined.

Material and methods

Chemicals

GlcNAc monohydrate (99%) and ManNAc monohydrate (99%) were obtained from Alfa Aesar (Karlsruhe, Germany). Enzymes used for cloning were purchased from New England Biolabs (Frankfurt, Germany). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were of analytical grade from various suppliers.

Bacterial strains and plasmids

E. coli strains DH5α (Invitrogen, Carlsbad, USA), BL21(DE3) and Tuner(DE3) (Novagen, Madison, USA), JM109(DE3) (Promega, Mannheim, Germany), C41(DE3) and C43 (DE3) (Lucigen, Middleton, USA) were used for cloning or overexpression experiments. The vectors used for the expression of AvaAGE are listed in Table 1.

Table 2
Sequences of oligonucleotides used in this study. Restriction sites are underlined.

Name	Sequence (5′ → 3′)	bp
Assembly PCR		
SBP-TEV_1	GAT GAA AAA ACC ACC GGC TGG CGC GGC GGC C	31
SBP-TEV_2	TTC GCC CGC CAG GCC TTC CAC CAC ATG GCC GCC GCG C	37
SBP-TEV_3	GCC TGG CGG GCG AAC TGG AAC AGC TGC GCG CGC GCC TG	38
SBP-TEV_4	TCG CGC TGG CCC TGC GGA TGA TGT TCC AGG CGC GCG CG	38
SBP-TEV_5	CAG GGC CAG CGC GAA CCG AGC GGC GGC TGC AAA CTG GGC G	40
SBP-TEV_6	GCC CTG AAA ATA CAG GTT TTC GCC CAG TTT GCA GC	35
Flanking_primer_f	GAT ACA GGA TCC GAT GAA AAA ACC ACC GGC TG	32
Flanking_primer_r	GAT ACA GGA TCC GCC CTG AAA ATA CAG GTT TTC G	34
Cloning oligonucleotides		
*avaage*_NdeI_f	GAT ACA CAT ATG GGG AAA AAC TTG CAA GCA TT	32
*avaage*_BamHI_r	GAT ACA GGA TCC TTA AGC TGA TTG TAA ACT CAA G	34
*avaage*_NcoI_f	GAT ACA CCA TGG GAG GGA AAA ACT TGC AAG CAT	33
*avaage*_EcoRI_r	GAT ACA GAA TTC TTA AGC TGA TTG TAA ACT CAA GG	35

The vector pBEN-SET2a-SBP-TEV was generated in this study. This plasmid is based on pBEN-SET2a and contains an additional streptavidin-binding peptide (SBP) that can be cleaved after expression by using a protease from the tobacco etch virus (TEV). The SBP-TEV-sequence was generated by assembly PCR using the Assembly PCR Oligo Maker tool [33]. The corresponding oligonucleotides are listed in Table 2. The Chaperone Plasmid Set containing the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTF16 was purchased from TaKaRa Bio, Inc. (Otsu, Japan).

Cloning

The gene encoding AvaAGE (GenBank Accession Number CP000117.1; region 4441642 to 4442820) was amplified from isolated genomic DNA derived from *Anabaena variabilis* ATCC 29413 by polymerase chain reaction. The corresponding primers are listed in Table 2. The gene was cloned into pET-28a via NdeI/BamHI, into pETM-41 via NcoI/BamHI, and into all other expression vectors via BamHI/EcoRI restriction sites. *E. coli* DH5α was used to propagate plasmids. Sequencing of the purified plasmids was carried out by Eurofins MWG Operon (Ebersberg, Germany). Unless stated otherwise, each purified plasmid was transformed into *E. coli* BL21(DE3) for protein expression.

Table 1
Vectors used for the expression of AvaAGE.

Vector	Tags used in this study	Origin
pET-28a(+)	Hexahistidine (His_6)	Novagen, Merck KgA, Darmstadt, Germany
pCOLADuet-1	His_6	
pETM-41	His_6-maltose-binding protein (His_6-MBP)	European Molecular Biology Laboratory (EMBL)
pGEX-4T-1	Glutathione-S-transferase (GST)	GE Healthcare, Piscataway, NJ
pBEN-SET1a	Solubility-enhancement tag 1 (SET1)	Stratagene (Agilent, Waldbronn, Germany)
pBEN-SET2a	Solubility-enhancement tag 2 (SET2)	
pBEN-SET3a	Solubility-enhancement tag 3 (SET3)	
pBEN-SET2a-SBP-TEV	SET2-SBP	This study
pET28b-SUMO-Ser	His_6-small ubiquitin-like modifier (SUMO)	Prof. M. Groll/Dr. F. Quitterer, Chair of Biochemistry, Technische Universität München, Germany

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