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New *N*-acyl-D-glucosamine 2-epimerases from cyanobacteria with high activity in the absence of ATP and low inhibition by pyruvate

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ABSTRACT

N-Acetylneuraminic acid, an important component of glycoconjugates with various biological functions, can be produced from *N*-acetyl-D-glucosamine (GlcNAc) and pyruvate using a one-pot, two-enzyme system consisting of *N*-acyl-D-glucosamine 2-epimerase (AGE) and *N*-acetylneuraminate lyase (NAL). In this system, the epimerase catalyzes the conversion of GlcNAc into *N*-acetyl-D-mannosamine (ManNAc). However, all currently known AGEs have one or more disadvantages, such as a low specific activity, substantial inhibition by pyruvate and strong dependence on allosteric activation by ATP. Therefore, four novel AGEs from the cyanobacteria *Acaryochloris marina* MBIC 11017, *Anabaena variabilis* ATCC 29413, *Nostoc* sp. PCC 7120, and *Nostoc punctiforme* PCC 73102 were characterized. Among these enzymes, the AGE from the *Anabaena* strain showed the most beneficial characteristics. It had a high specific activity of $117 \pm 2\,U\,mg^{-1}$ at 37 °C (pH 7.5) and an up to 10-fold higher inhibition constant for pyruvate as compared to other AGEs indicating a much weaker inhibitory effect. The investigation of the influence of ATP revealed that the nucleotide has a more pronounced effect on the K_m for the substrate than on the enzyme activity. At high substrate concentrations (≥200 mM) and without ATP, the enzyme reached up to 32% of the activity measured with ATP in excess.

1. Introduction

N-Acetylneuraminic acid (Neu5Ac) is the most common member of the family of sialic acids, which frequently occupy the terminal position of glycan chains present on many eukaryotic glycoproteins and glycolipids (Angata and Varki, 2002; Schauer, 1991). Due to their exposed position, sialic acids are involved in diverse cellular processes. Amongst others, they interact with sialic-acid-recognizing lectins mediating cell–cell interactions and signaling, affect the malignancy of cancer cells, and serve as recognition sites for viruses and infectious microorganisms (Schauer, 2009; Varki, 2008).

Due to its role as binding site for viruses and its cellular signaling function, Neu5Ac is an important starting material for the production and development of pharmaceutical agents (Tao et al., 2010; von Itzstein, 2007). Neu5Ac can be synthesized from pyruvate and *N*-acetyl-D-mannosamine (ManNAc) in a reversible aldol condensation reaction catalyzed by an *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3). This reaction has been extensively studied during the last decades (Kim et al., 1988; Mahmoudian et al., 1997; Groher and Hoelsch, 2012). Although most studies focused on reaction systems with isolated enzymes (e.g. Kragl et al., 1991; Maru et al., 1998), approaches using engineered whole-cells (Kang et al., 2012; Tao et al., 2011; Xu et al., 2007; Zhang et al., 2010) or spore surface displayed NAL are emerging (Gao et al., 2011; Xu et al., 2011). The synthesis suffers from an unfavorable position of the thermodynamic equilibrium leading to an incomplete use of raw materials (Comb and Roseman, 1960). To push the reaction toward the product side, an excess of pyruvate is used (Kim et al., 1988). Additionally, the reaction is performed at low temperatures – typically in the range of 20–30 °C – because the equilibrium constant increases with decreasing temperature (Kragl et al., 1991; Liese et al., 2006).

Since ManNAc is an expensive compound and not readily available in large amounts, it is generated from the lower priced *N*-acetyl-D-glucosamine (GlcNAc) by epimerization at the C-2 position. This reaction can be catalyzed either enzymatically by an *N*-acyl-D-glucosamine 2-epimerase (AGE, EC 5.3.1.8) (Kragl et al., 1991; Maru et al., 1998) or chemically under alkaline conditions (Mahmoudian et al., 1997; Blayer et al., 1999; Xu et al., 2007). The one-pot, two-enzyme system has been proposed to be more simple, cost-effective, and environmentally sustainable than the chemo-enzymatic method for the production of Neu5Ac (Maru et al., 2002;

Abbreviations: AGE, *N*-acyl-D-glucosamine 2-epimerase; GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; NAL, *N*-acetylneuraminate lyase; Neu5Ac, *N*-acetylneuraminic acid; TCA, trichloroacetic acid.

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Tao et al., 2010). This is because the chemo-enzymatic route is carried out in two steps with intermediate product isolation and involves large amounts of strong bases and organic solvents, which are needed for the epimerization and product purification, respectively (Liese et al., 2006; Tao et al., 2010). Nevertheless, a major part of the industrial Neu5Ac production still uses a preceding chemical epimerization step (Liese et al., 2006). This is mainly due to unfavorable properties of currently known AGEs.

Several AGEs from different sources have been biochemically characterized: the eukaryotic enzymes from humans (Takahashi et al., 1999; Lee et al., 2004), pigs (Lee et al., 2007a,b), and rats (Takahashi et al., 2001, 2002) as well as the prokaryotic AGEs from *Bacterioides ovatus* ATCC 8483 (Sola-Carvajal et al., 2012) and from the cyanobacteria *Anabaena* sp. CH1 (Lee et al., 2007a,b) and *Synechocystis* PCC 6803 (Tabata et al., 2002). In addition, the kinetic parameters of a commercially available enzyme from unknown source have been determined (Zimmermann et al., 2007). The eukaryotic AGEs characterized so far show some disadvantages for the large-scale production of Neu5Ac. Namely, they were found to have relatively low specific activities, to be inhibited by pyruvate, which is a severe problem for the one-pot synthesis, and to have a strong dependence on ATP as allosteric activator. The AGE from porcine kidney, which has been most commonly used for preparative purposes (Liese et al., 2006; Maru et al., 1998), has a specific activity of 32.5 U mg^{-1} at 37 °C and a half-saturation constant for ATP ($K_{m,ATP}$) of 180 μM (Lee et al., 2007a; Maru et al., 1996). For the production of Neu5Ac using the porcine AGE at the 150 L-scale, 910 g of ATP were added to the reaction mixture, which corresponds to a concentration of 10 mM (Maru et al., 1998). Although ATP is not consumed during the reaction, it is expensive and easily cleaved by contaminating hydrolases in an enzyme preparation. For these reasons, it has a strong influence on the cost-effectiveness of the process (Tao et al., 2010).

Cyanobacteria represent a valuable source of enzymes with interesting properties due to their unique physiological features and remarkable biodiversity (Fu et al., 2013; Hölsch and Weuster-Botz, 2010). Especially the AGE from *Anabaena* sp. CH1, which has been discovered by Lee et al. (2007a), showed superior attributes when compared to their eukaryotic counterparts. This enzyme has a specific activity of 123.8 U mg^{-1} at 37 °C, which is almost 4-fold faster than the porcine AGE. Furthermore, with a $K_{m,ATP}$ of 1–2 μM, the ATP-requirement of this enzyme was approximately 100-fold lower with respect to the porcine AGE. Still, the *Anabaena* sp. CH1 AGE was shown to be susceptible to pyruvate. At a concentration of 200 mM, the enzyme activity was reduced by 50% (Lee et al., 2007a).

Hence, in this study, four novel cyanobacterial AGEs derived from *Acaryochloris marina* MBIC 11017, *Anabaena variabilis* ATCC 29413, *Nostoc* sp. PCC 7120, and *Nostoc punctiforme* PCC 73102 were recombinantly expressed in *Escherichia coli* and were biochemically characterized for their epimerization of GlcNAc to ManNAc in terms of suitability for Neu5Ac production.

2. Materials and methods

2.1. Chemicals

GlcNAc monohydrate (99%) and ManNAc monohydrate (99%) were purchased from Alfa Aesar (Karlsruhe, Germany). ATP (≥98%) and sodium pyruvate (≥99%) were purchased from Carl Roth (Karlsruhe, Germany). Enzymes used for DNA work were obtained from New England Biolabs (Frankfurt, Germany). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were of analytical grade from various suppliers.

2.2. Bacterial strains and plasmids

E. coli strains DH5α (Invitrogen, Carlsbad, USA) and BL21(DE3) (Novagen, Madison, USA) were used for cloning and overexpression experiments, respectively. The plasmid pET28a(+) (Novagen, Madison, USA) served as expression vector.

2.3. Cloning

The genes encoding the respective AGE were amplified from isolated genomic DNA derived from *A. marina* MBIC 11017, *A. variabilis* ATCC 29413, *Nostoc* sp. PCC 7120, and *N. punctiforme* PCC 73102 by polymerase chain reaction. The corresponding primers were designed according to available gene sequences (Supplementary Material, Table S1) and are listed in table S2 (Supplementary Material). Each amplified gene was digested with NdeI and BamHI and was ligated into a linearized pET28a(+) vector. The vector constructs included an N-terminal hexahistidine-tag (His_6-tag) in frame with the cloned gene. *E. coli* DH5α were transformed with each vector for selection and plasmid propagation. Sequencing of the purified plasmids was carried out by Eurofins MWG Operon (Ebersberg, Germany). For protein expression, each purified plasmid was transformed into *E. coli* BL21(DE3).

2.4. Protein expression and purification

For each AGE, a single *E. coli* BL21(DE3) colony carrying the respective plasmid was used as inoculum for a 4 mL preculture (LB-medium supplemented with 30 mg L^{-1} kanamycin in 15 mL test tubes). The preculture was grown over night at 30 °C and 200 rpm, 2.5 cm excentricity. One milliliter of the preculture was used to inoculate a 1 L shaking flask without baffles containing 200 mL of LB-medium supplemented with 30 mg L^{-1} kanamycin. The culture was incubated at 37 °C and 250 rpm, 5 cm excentricity. The cells were grown to an OD at 600 nm of 0.8. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Subsequently, the cells were incubated for 16 h at 20 °C and 250 rpm, 3.5 cm excentricity. The collection and the mechanical lysis of the cells as well as the purification procedure using a 1 mL HisTrap FF crude column (GE Healthcare, Uppsala, Sweden) were performed according to Groher and Hoelsch (2012). After purification, the buffer was changed to the respective reaction buffer by dialysis. If not stated otherwise, the reactions were performed in 0.1 M sodium phosphate buffer (pH 7.5). Protein purity was judged by SDS-PAGE stained with Coomassie. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA) using bovine serum albumin (BSA) as standard.

2.5. Determination of kinetic parameters

The cyanobacterial AGEs were characterized for their epimerization of GlcNAc to ManNAc by initial rate measurements. All assays were performed in 1.5 mL safe lock tubes using a thermal shaker. Enzyme concentrations ranged from 1 to 20 μg mL^{-1} (20–420 nM). Negative controls without enzyme were routinely included. To determine enzyme kinetic parameters, triplicate measurements at 8–12 different GlcNAc concentrations spanning at least 0.3–5 × K_m were performed. If not stated otherwise, the ATP concentration was kept constant at 1 mM. For the determination of kinetic parameters in the absence of ATP, GlcNAc concentrations up to 640 mM were used. To investigate the inhibiting effect of pyruvate on AGEs, it was added at concentrations of up to 400 mM. Half-saturation constants for ATP were determined in a concentration range of 0–25 μM at constant 200 mM GlcNAc.