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Note

Simple and large-scale production of *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and pyruvate using *N*-acyl-D-glucosamine 2-epimerase and *N*-acetylneuraminate lyase

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Abstract

N-Acetylneuraminate lyase and *N*-acyl-D-glucosamine 2-epimerase had been cloned and over-expressed in *Escherichia coli*. Simultaneous use of these two enzymes and feeding of appropriate amounts of pyruvate to the reaction mixture made possible the high conversion of *N*-acetylneuraminic acid (Neu5Ac) from *N*-acetyl-D-glucosamine (GlcNAc) with a 77% conversion rate on a molar basis. As a result, 29 kg of Neu5Ac was obtained from 27 kg of GlcNAc. The product was recovered by direct crystallization, and verified as identical to authentic Neu5Ac.

Keywords: *N*-acetyl-D-glucosamine; *N*-acetyl-D-mannosamine; *N*-acetylneuraminate lyase; *N*-acyl-D-glucosamine 2-epimerase; Sialic acid

N-Acetylneuraminic acid (Neu5Ac), the most ubiquitous species amongst the sialic acids, is widely distributed in the animal kingdom as a component of oligosaccharides, glycoproteins, and glycolipids. Neu5Ac carries out various biological functions by acting as receptors for microorganisms, viruses, toxins, and hormones, by masking receptors and by regulation of the immune system [1]. Neu5Ac has been attracting glycobiologist's interests because of its versatile biological functions, and has stimulated the development of new types of therapeutics [2].

Neu5Ac has been prepared hitherto either from colominic acid (Neu5Ac homopolymer through α-2,8-linkage) [3] or from natural resources such as edible birds nest (petrel), milk or egg [4]. However, the drawbacks of those conventional methods make them unsuitable for large scale Neu5Ac production. On the other hand, enzymatic synthesis of Neu5Ac from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate has been described with *N*-acetylneuraminate lyase acid lyase (Neu5Ac lyase) as catalyst [5,6]. Because of the expense and difficulty

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of large-scale preparation of ManNAc, a method for preparing Neu5Ac by reacting inexpensive *N*-acetyl-D-glucosamine (GlcNAc) and pyruvic acid in the presence of Neu5Ac lyase and *N*-acyl-D-glucosamine 2-epimerase (GlcNAc 2-epimerase), the latter of which catalyzes the conversion of GlcNAc to ManNAc has been examined (Fig. 1). Kragl *et al.* reported a method for the synthesis of Neu5Ac by using the GlcNAc 2-epimerase and Neu5Ac lyase in dissolved form in the enzyme membrane reactor [7]. The fatal defect of this method for large scale production of Neu5Ac was provision of GlcNAc 2-epimerase in efficient amount for an unrestricted scale of reaction. GlcNAc 2-epimerase has been found to occur in some mammalian organs in trace amounts, not applicable for industrial use.

1. Results and discussion

In order to mass-produce GlcNAc 2-epimerase, we have cloned its gene from porcine kidney [8]. *Escherichia coli* cells transformed with the gene overexpressed the GlcNAc 2-epimerase having the same properties as those of the GlcNAc 2-epimerase from porcine kidney. Neu5Ac lyase from *E. coli* K-12 had been cloned and overexpressed in *E. coli*, too [9]. Combining these two enzymes, it was possible to produce Neu5Ac directly from GlcNAc and pyruvate on an industrial scale.

The enzymatic epimerization and aldol condensation proceeds in a reversible process. Therefore, high concentrations of GlcNAc lead to high ManNAc concentration, which shifts the equilibrium position of the second reaction toward Neu5Ac. Since the equilibrium of Neu5Ac lyase lies between Neu5Ac and pyruvate/ManNAc, excess molar amounts of pyruvate (up to five-fold) is usually used to shift the equilibrium to the synthesis of Neu5Ac. However, excess amounts of pyruvate inhibits GlcNAc 2-epimerase, resulting in inefficient formation of ManNAc and requirement of removal of large amounts of residual pyruvate when isolating Neu5Ac produced in reaction solution [10]. Here, we report a simple enzymatic system for a large scale production of Neu5Ac from GlcNAc and pyruvate by using GlcNAc 2-epimerase and Neu5Ac lyase.

As preliminary experiments, small scale and kinetic investigations were carried out to determine the conditions for production of Neu5Ac using the two enzymes (data not shown). Based on the data obtained from preliminary experiments, a reaction solution was prepared by dissolving 27 kg (122 mol) of GlcNAc and 8 kg (73 mol) of pyruvate in 150 L of water before which GlcNAc 2-epimerase and Neu5Ac lyase were added. The ratio of GlcNAc and pyruvate at the initial reaction was 1:0.6 on a molar basis. A high concentration of GlcNAc and pyruvate prevented loss of enzyme activity and contamination by microorganisms. The GlcNAc 2-epimerase required ATP and Mg^{2+} for activation. Since both enzymes can be used in a pH range of 7.0 to 8.0 without loss of activity, the pH in the reactor was adjusted to 7.2 with NaOH.

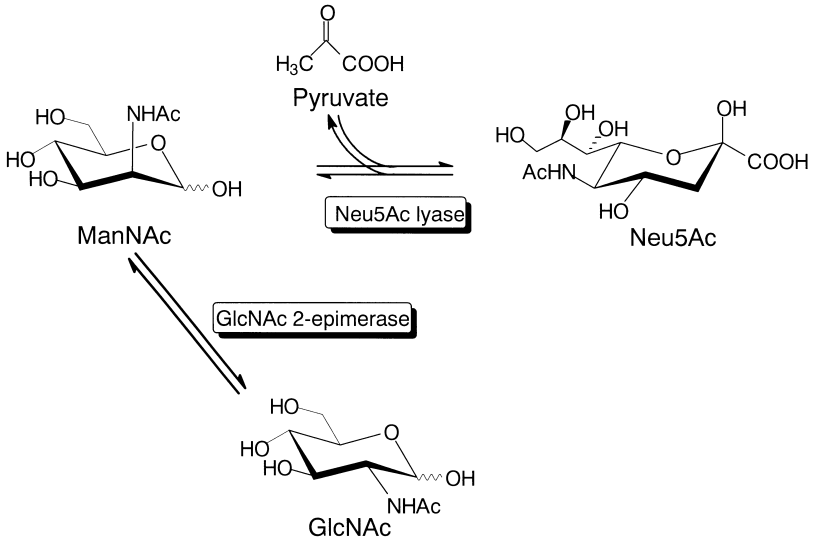

Fig. 1. Synthesis of Neu5Ac from GlcNAc and pyruvate

Buffers were completely unnecessary. The reaction was started by the addition of GlcNAc 2-epimerase and Neu5Ac lyase. The time-course for the synthesis of Neu5Ac is shown in Fig. 2.

After 50 h at 30 °C, the reaction reached equilibrium with a conversion rate from GlcNAc to Neu5Ac of 40% on a molar basis. In order to shift the equilibrium to the synthesis of Neu5Ac in the reaction mixture, a further 12.5 kg of sodium pyruvate was added. The ratio of the initial amounts of GlcNAc and total amount of pyruvate was 1:1.5. After 140 h, the reaction reached to equilibrium at which the conversion rate from GlcNAc to Neu5Ac was 68%. Seven kg sodium pyruvate was further fed into the reaction mixture, the ratio of GlcNAc and pyruvate being 1:2. After 240 h of the reaction, the conversion rate from GlcNAc to Neu5Ac reached to 77%. In this reaction, 84% of GlcNAc was converted to 77% of Neu5Ac and 7% of ManNAc. Feeding of appropriate amounts of pyruvate in the reaction mixture led to higher conversion from GlcNAc to Neu5Ac. The product was recovered by direct crystallization. After heating for 5 min at 80 °C, the reaction mixture was filtered to remove insoluble materials such as denatured proteins. To recover Neu5Ac from the reaction mixture, crystallization was initiated by adding 5 volumes of glacial acetic acid. The crystalline Neu5Ac was washed with ethanol to remove residual acetic acid, and then dried at 40 °C until a constant weight was obtained. As a result, 23 kg (74 mol) of Neu5Ac was obtained from 27 kg (122 mol) of GlcNAc. The retention time determined by high performance liquid chromatographic (HPLC) analysis and infrared (IR) spectrum of isolated crystalline Neu5Ac were indistinguishable from those of authentic Neu5Ac.

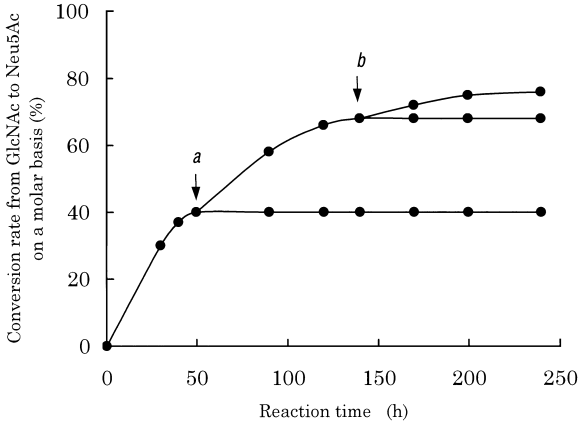

Fig. 2. Time-course of fed-batch reaction for the synthesis of Neu5Ac from GlcNAc and pyruvate. Arrows show the point of feeding with (a) 37.9 L and (b) 21.6 L solutions of sodium pyruvate (12.5 kg and 7 kg, respectively).

2. Experimental

Preparation of Neu5Ac.—Substrates of 27 kg of GlcNAc and 8 kg of sodium pyruvate were added to a 200 L of batch reactor, and dissolved with 150 L of deionized water. After 910 g of ATP and 305 g of $MgCl_2$ were added to the substrate mixture and the pH was adjusted to 7.2 with NaOH. The reaction was started by the addition of 1.2×10^6 units of Neu5Ac lyase and 3×10^5 units of GlcNAc 2-epimerase. The temperature was maintained at 30 °C. Periodically, 10 mL samples were removed, and Neu5Ac in the reaction mixture was determined by thiobarbituric acid (TBA) method [11]. When Neu5Ac synthesis reached to first plateau, 37.9 L of sodium pyruvate (12.5 kg) was added and the incubation continued under the same conditions. At the second plateau, 21.2 L of sodium pyruvate (7 kg) was added. When the highest yield of Neu5Ac was obtained, the reaction was stopped by heating at 80 °C for 5 min. Insoluble materials appearing were removed by vacuum filtration using filter paper No.2 (Whatman Ltd.). (Neu5Ac in the reaction mixture, 29 kg; corresponding to 77% of GlcNAc on a molar basis) The reaction mixture was concentrated to one-third of its initial volume in a rotary evaporator below 40 °C *in vacuo*. The Neu5Ac in the reaction mixture was crystallized by adding five volumes of glacial acetic acid to concentrated solution. After standing for one day at 4 °C, the solid was recovered by vacuum filtration using filter paper No.2 and washed with ethanol before drying at 40 °C *in vacuo* to constant weight. (Neu5Ac, 23 kg; 61% yield based on GlcNAc used on a molar basis).

Analysis of Neu5Ac, GlcNAc, ManNAc, and pyruvate.—Neu5Ac was determined by TBA method [11]. GlcNAc and ManNAc in the reaction mixture were treated with l-phenyl-3-methyl-5-pyrazolone, and the derivatives were analyzed by HPLC [12]. HPLC analysis of Neu5Ac was performed with a Shimadzu LC-6A (Shimadzu Corporation) pump and a column of Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories). The mobile phase was 10 mM H_2SO_4 and the flow-rate was 0.5 mL/min. The elution profile was monitored by UV absorbance at 205 nm. The IR spectrum of Neu5Ac was recorded

with a Shimadzu FTIR-8100M spectrometer (Shimadzu Corporation).

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