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Short communication

A capillary electrophoresis method with dynamic pH junction stacking for the monitoring of cerebroside sulfotransferase

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ABSTRACT

Metachromatic leukodystrophy (MLD) is a rare and severe genetic disease. Inhibition of cerebroside sulfotransferase (CST) has been proposed as a promising new therapeutic strategy for the treatment of MLD. CST catalyzes the transfer of a sulfate group from the coenzyme 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to cerebroside yielding cerebroside sulfate and adenosine-3′,5′-diphosphate (PAP). So far only a few weak CST inhibitors have been described. The goal of the present study was to establish a suitable assay for identifying and characterizing novel CST inhibitors. To this end, we developed and optimized a capillary electrophoresis (CE) based assay for monitoring the catalytic activity of CST by measuring the formation of PAP. A sample matrix consisting of 5 mM phosphate buffer with about 0.0001% polybrene at pH 7.4 and a background electrolyte (BGE) containing 75 mM phosphate buffer with 0.002% polybrene at pH 5.6 were utilized to achieve a stacking effect for PAP by dynamic pH junction. This led to a limit of detection for the enzymatic product PAP of 66.6 nM. The CE method was sensitive, robust, and suitable for CST inhibitor screening, K_i value determination, and enzyme kinetic studies. Selected reference compounds were tested in order to validate the assay, including the substrate s cerebroside and psychosine, and the inhibitor Congo Red. The newly developed CE method will be useful for the identification and development of novel CST inhibitors which are urgently needed for the treatment of MLD.

1. Introduction

The enzyme cerebroside sulfotransferase (CST) is a promising target for metachromatic leukodystrophy (MLD), which is a rare genetic disease characterized by a dysfunction of the enzyme arylsulfatase A. This defect leads to the lysosomal accumulation of cerebroside sulfate (sulfatide), which causes a progressive destruction of white matter throughout the nervous system [1–3]. Potential therapeutic options for MLD include enzyme replacement, substrate reduction, stem cell transplantation, and gene therapy [1,4]. CST catalyzes the last step in the synthesis of cerebroside sulfate (sulfatide), namely the transfer of a sulfate group from the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to cerebrosides (Fig. 1) [5]. Our aim is to develop CST inhibitors which will reduce the biosynthesis of sulfatide and as a consequence prevent, delay or reduce sulfatide aggregation in the central and peripheral nervous system [5].

The commonly used method for the monitoring of CST activity is a radioisotopic assay monitoring the transfer of radioactive sulfate from [^{35}S]-labelled 3′-phosphoadenosine-5′-phosphosulfate ([^{35}S]PAPS) to cerebroside sulfate (sulfatide), the product of CST; [^{35}S]cerebroside sulfate is then separated by thin-layer or paper chromatography [6–14]. The drawback of the radioisotopic assay includes the high price of [^{35}S]PAPS and the lengthy procedure associated with planar chromatography. The main challenge of monitoring sphingolipids is the quantification of all sphingolipid species. Therefore, a straightforward method suitable for compound screening, which would allow the direct measurement and quantification of a non-lipid product of the enzymatic reaction would be highly desirable.

We have now developed a simple, economic, and highly sensitive capillary electrophoresis (CE) method for the monitoring of CST activity via quantification of the enzymatic product adenosine-3′,5′-diphosphate (PAP) (Fig. 1). In order to enhance the detection sensitivity of PAP, we applied dynamic pH junction stacking. Our new CE method enables an efficient separation of the components in the enzyme reaction mixture and allows for enzyme kinetic studies, compound library screening, and inhibitor characterization.

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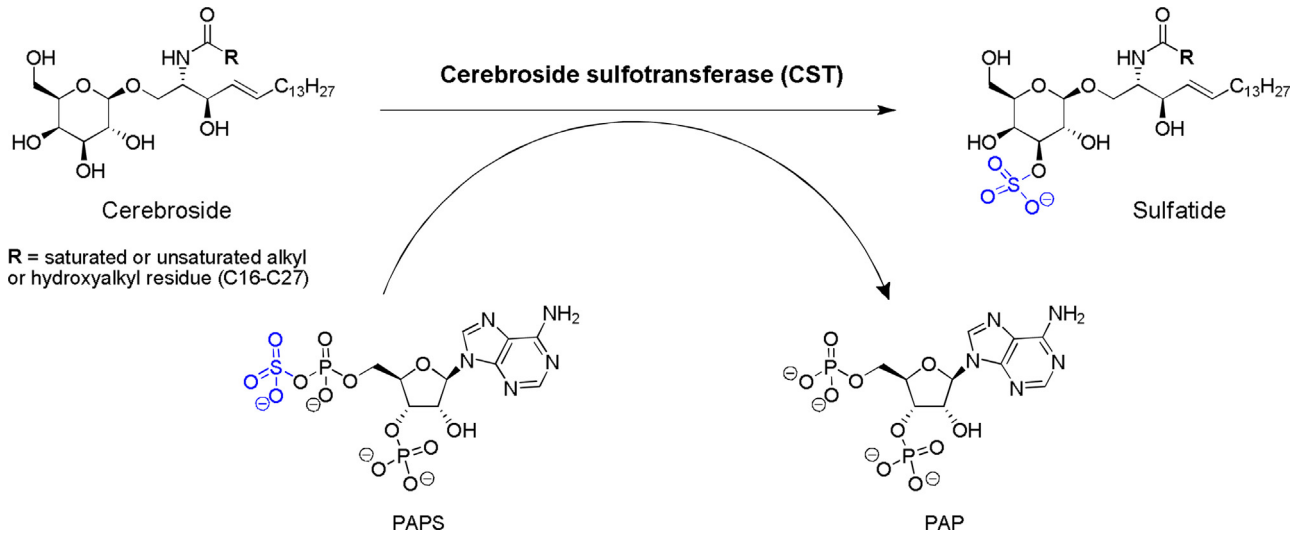

Fig. 1. Reaction catalyzed by cerebroside sulfotransferase (CST) [5]. CST catalyzes the conversion of cerebroside to sulfatide. The co-substrate PAPS acts as a sulfate donor and is converted to PAP.

2. Material and methods

2.1. Apparatus and CE measurements

All experiments were carried out by using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a DAD detector. The capillary temperature was kept constant at 15 °C. The electrophoretic separations were carried out by using a fused-silica capillary of 60 cm total length (50 cm effective length) × 75.5 μm (id) × 363.7 μm (od) obtained from Optronis GmbH. The following conditions were applied: λ_{max} = 260 nm, voltage = −15 kV, running buffer 75 mM phosphate buffer, 0.002% polybrene, pH 5.6 (adjusted by phosphoric acid), and electrokinetic injection (−10 kV, 30 s). The capillary was washed with 0.2 M NaOH for 2 min, and subsequently with running buffer for 2 min before each injection. Data collection and corrected peak area analysis were performed by the 32 Karat software obtained from Beckman coulter (Fullerton, CA, USA). Further data analysis was carried out by Graph Pad Prism 4 (Graph Pad Software, Inc., CA, USA) and Excel.

2.2. Chemicals

Hexadimethrine bromide (polybrene), Congo Red, guanosine 5′-diphosphate (GDP), psychosine, Triton X-100, and magnesium chloride were obtained from Sigma (Steinheim, Germany). Cerebroside was obtained from Matreya LLC (Pleasant Gap, PA, USA); according to the supplier it consists of a mixture of cerebrosides with saturated or unsaturated alkyl residues (C16:0, C18:0, C20:0, C22:0, C23:0, C24:0–C27, C24:1–C27:1) or hydroxyalkyl residues (C18:0(2–OH), C20:0(2–OH), C22:0(2–OH), C23:0(2–OH), C24:0(2–OH), C24:1(2–OH), C25:1(2–OH)). 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) was obtained from AppliChem and disodium hydrogenphosphate dihydrate was obtained from Roth. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) was purchased from Bellbrook Labs (No. 2059) in high purity. Other commercial sources of PAPS typically contain significant amounts of PAP and are therefore not suitable for the assay.

2.3. Buffers

Reaction buffer. The enzyme reaction buffer contained 10 mM HEPES and 16 mM $MgCl_2$. The buffer was adjusted to pH 7.1 at 37 °C by addition of aq. NaOH solution.

Running buffer. The CE running buffer consisted of 75 mM phosphate buffer pH 5.6 containing 0.002% polybrene. The pH value was adjusted by the addition of phosphoric acid to pH 5.6. The resulting mixture was treated in an ultrasonic bath for 5 min.

Stacking buffer. The stacking buffer consisted of a 5 mM phosphate buffer pH 7.4, containing about 0.0001% polybrene. It was prepared by a 1:15 dilution of 75 mM phosphate buffer pH 7.4 containing 0.002% polybrene.

2.4. Enzyme expression

The human CST enzyme was obtained by heterologous expression in CHO cells in analogy to a described procedure [14].

2.5. Procedure

2.5.1. Enzymatic reactions of CST

CST-catalyzed reactions were carried out in a total volume of 50 μl containing PAPS and cerebrosides (concentrations of PAPS and cerebrosides varied according to assay type) in 10 mM HEPES reaction buffer containing 16 mM $MgCl_2$, pH 7.1. Lipids and Triton X-100 were dissolved in chloroform:methanol (1:1). Triton X-100 (10 μl of a 1% solution) and the required amount of lipid solution were pipetted into the reaction vials, and the organic solvents chloroform and methanol were removed by drying before the reaction buffer was added. Reactions were initiated by the addition of 938 ng of human CST, followed by incubation at 37 °C for 30 min. All enzymatic reactions were stopped by heating for 10 min at 60 °C.

2.5.2. Sample preparation and method validation

After stopping of the CST reaction by heating, 50 μl of a 20 μM GDP stock solution in water were added to the reaction vial as an internal standard (IS). The resulting mixture was further diluted 1:4 with stacking buffer (phosphate buffer, 5 mM, pH 7.4, containing about 0.0001% polybrene). Then 90 μl of the diluted mixture