ELSEVIER

Journal of Chromatography B, 696 (1997) 193–202

JOURNAL OF CHROMATOGRAPHY B

Improved high-performance liquid chromatographic method for N-acetylgalactosamine-4-sulfate sulfatase (arylsulfatase B) activity determination using uridine diphospho-N-acetylgalactosamine-4-sulfate

Antoni J. Leznicki[a,*], Karol Bialkowski[b]

[a]*Department of Biochemistry, Institute of Biology and Environmental Sciences, Nicholas Copernicus University, PL 87-100 Torun, Poland*

[b]*Department of Clinical Biochemistry, University School of Medical Sciences, PL 85-092 Bydgoszcz, Poland*

Received 28 January 1997; revised 18 April 1997; accepted 18 April 1997

Abstract

UDP-N-acetylgalactosamine-4-sulfate (UDP-GalNAc-4-S) was isolated from hen oviduct (isthmus) with a yield of 31 μmol per 100 g of wet tissue and used for arylsulfatase B (ASB) activity determination. Two HPLC methods of separation and quantitation of the reaction product were described: (1) an original gradient elution method which makes it possible to determine the reaction product when only partially purified ASB was used and additional uridine derivatives were formed during incubation; (2) an improved, fast isocratic elution method which may be used in the case of purified ASB preparations, devoid of other nucleotide hydrolysing enzymes. For both methods the detection limit was 0.1 nmol of product with standard error of determination ≤3%. Using the gradient elution method we have found that UDP-GalNAc-4-S was hydrolysed by bovine arylsulfatase B1 most efficiently at pH 5.0 and concentration 0.5 m*M* with K_m=85 μ*M*.

Keywords: Enzymes; N-Acetylgalactosamine-4-sulfate sulfatase; Arylsulfatase B; Uridine diphospho-N-acetylgalactosamine-4-sulfate

1. Introduction

Lysosomal arylsulfatases which are in fact sugar sulfate sulfohydrolases exist as two distinct enzyme groups of different physiological function. Arylsulfatase A (ASA) is cerebroside-3-sulfatase and inherited deficiency of the enzyme activity in humans results in genetic disease, metachromatic leucodystrophy (for review see Ref. [1]). Arylsulfatase B (ASB) is involved in desulfation of 4-sulfate groups occurring at the non-reducing terminal N-acetylgalactosamine-4-sulfate of oligosaccharides derived from dermatan sulfate or chondroitin-4-sulfate [2–4]. The lack of the enzyme activity is the cause of mucopolysaccharidosis VI (Maroteaux–Lamy syndrome) [5]. There are several problems regarding independent determination of the activity of both enzymes [6–8] with commonly used artificial sub-

*Corresponding author. Temporary address for correspondence: Michigan State University, Department of Botany and Plant Pathology, Rm. 339, East Lansing, MI 48824, USA.

PII S0378-4347(97)00263-6

strates 4-nitrocatechol sulfate or 4-methylumbelliferone sulfate [9]. Arylsulfatases A and B in mammals exhibit similar specificity towards both substrates but they differ in molecular features like molecular mass and isoelectric point. Both groups of enzymes can be completely separated each other using Blue-Sepharose affinity chromatography [10] or partially resolved on DEAE-cellulose [5,8,10–12]. Arylsulfatases of lower vertebrates and invertebrates exist in various molecular forms which were not so extensively investigated and characterized. Their molecular and kinetic properties are often intermediate between both A and B mammalian arylsulfatases [13,14], so it is difficult to establish their relationship to mammalian enzymes. For this reason it is necessary to determine their activity towards the appropriate natural substrates. The labelled natural substrates were most commonly used for this purpose [15–21]. Due to necessity of enzymatic synthesis of [^{35}S]-labelled substrates like dermatan sulfate, chondroitin-4-sulfate-derived oligosaccharides or UDP-N-acetylgalactosamine-4-sulfate (UDP-GalNAc-4-S) determination of ASB activity is rather difficult [15,16,18,19,21]. Fluharty et al. have reported the alternative procedure for assay of the arylsulfatase B activity toward UDP-GalNAc-4-S based on HPLC determination of reaction product [22]. In this paper we described the improvements on the preparation of the substrate and the method for ASB activity determination. Also, the pitfalls and limitations of the above method are discussed.

2. Experimental

2.1. Chemicals

Tris(hydroxymethyl)aminomethane, UDP-N-acetylgalactosamine (UDPGalNAc), methanol, α-methyl-D-glucopyranoside, tetrabutylammonium hydroxide, ammonium acetate and acetic acid were from Sigma (St.Louis, MO, USA). Dowex AG1×10 (200–400 mesh) was purchased from BioRad (Hercules, CA, USA) and charcoal Darco G60 from Serva (Heidelberg, Germany). Blue-Sepharose CL 4B and ConA-Sepharose CL 4B have been synthesized in our laboratory according to general procedure. Phenyl-Sepharose CL 4B was bought from Pharmacia Fine Chemicals (Uppsala, Sweden) and DEAE-cellulose from Whatman (Maidstone, UK). The artificial substrate, 2-hydroxy-5-nitrophenyl sulfate dipotassium salt (NCS) was obtained by the method of Roy [23]. All other chemicals were of the highest purity available and were purchased from the Polish Chemical Reagents (POCh) (Gliwice, Poland).

2.2. Isolation of uridine diphospho-N-acetylgalactosamine-4-sulfate

The natural substrate, UDP-GalNAc-4-S, was extracted from the isthmus part of the hen oviduct [24,25] and purified by the modified method of Donovan et al. [26]. Briefly, 100 g of isthmi were excised from the hen oviducts and homogenized with 150 ml redistilled water. The resulting homogenate was boiled for 5 min, chilled to room temperature and centrifuged (20 min, 15 000 *g*). The pellet was rehomogenized in 100 ml of 10 m*M* ammonium formate and centrifuged again. Both supernatants were combined and applied to the 3.5×10 cm Dowex AG1×10 (formate) column. Elution of unadsorbed material was performed with 50 ml 10 m*M* ammonium formate and 100 ml redistilled water. Adsorbed nucleotides were eluted with linear formate gradient (A: 250 ml of 4 *M* formic acid, B: 250 ml of 1 *M* ammonium formate in 4 *M* formic acid). The flow-rate was 0.5 ml/min and 6 ml fractions were collected. The fractions corresponding to the last eluted peak were pooled, 2 g of active charcoal Darco G60 were added, stirred for 2 h and then vacuum filtered on the Buchner funnel. The charcoal sediment was washed with 100 ml of 1 m*M* HCl and then adsorbed UDP-GalNAc-4-S was eluted with three 50 ml portions of 30 m*M* ammonium hydroxide in 50% ethanol. Elution of the compound was monitored at 260 and 280 nm. The eluate was evaporated under vacuum at 35°C and the solid residue was dissolved in 3 ml of water. A concentrated preparation was filtered through 0.22 μm Nylon membrane and the nucleotide concentration was estimated spectrophotometrically by using a molar absorption coefficient of 10 000 M^{-1} cm^{-1} at 262 nm. The preparation was divided into small aliquots and stored at −20°C.

2.3. Purification of arylsulfatases

Arylsulfatases A and B were isolated from bovine liver. The fresh tissue was homogenized with 9 volumes of 20 m*M* Tris–HCl buffer, pH 7.2 containing 0.1% Triton X-100. The homogenate was centrifuged 60 min at 3000 *g* and the enzyme activity in supernatant was precipitated with acetone in the range of concentration 20–65% (v/v) at a temperature of 0 to −15°C. The precipitate was dissolved in 20 m*M* Tris–HCl buffer, pH 7.2 and centrifuged to remove the insoluble residue. The resulting supernatant was applied to the DEAE-cellulose (DE-32) column. Elution of the unbound ASB1 was carried out with starting buffer (20 m*M* Tris–HCl buffer, pH 7.2) and adsorbed ASB2 and ASA were eluted with linear NaCl concentration gradient (0–0.8 *M* in the starting buffer). The fractions with ASB1 activity were pooled and directly applied to the Blue-Sepharose CL 4B column equilibrated with 50 m*M* Tris–acetate buffer, pH 6.0. The column was washed with equilibration buffer and arylsulfatase activity was eluted with the same buffer containing 0.5 *M* NaCl.

ASA preparation after DEAE-cellulose chromatography and ASB1 after Blue-Sepharose chromatography were further purified on ConA-Sepharose CL 4B column equilibrated with 50 m*M* Tris–HCl buffer, pH 6.0 containing 0.5 m*M* each $MnCl_2$, $CaCl_2$, $MgCl_2$ and 0.5 *M* NaCl. Arylsulfatase activities were eluted from the column with 20% α-methyl-D-glucopyranoside in the starting buffer. The part of ASB1 preparation was concentrated by ultrafiltration with buffer exchange to 20 m*M* Tris–HCl, pH 7.2 and used in natural substrate hydrolysis experiments.

The rest of the arylsulfatase B1 and arylsulfatase A were finally purified by hydrophobic interaction chromatography on Phenyl-Sepharose CL 4B in the same way. To the enzyme preparation was added NaCl up to 3 *M* concentration and it was applied to the column equilibrated with 50 m*M* Tris–HCl buffer, pH 7.4 containing 3 *M* NaCl. The column was washed consecutively with the starting buffer, 50 m*M* Tris–HCl buffer, pH 7.2+0.5 *M* NaCl and 25 m*M* Tris–HCl buffer, pH 7.2. Thereafter, the column was eluted with a linear gradient of ethylene glycol (0–30%, v/v) in the latter buffer. The arylsulfatase B1 preparation termed as "purified ASB1" was concentrated on Amicon YM10 membrane with buffer exchange for 20 m*M* Tris–HCl, pH 7.2. Arylsulfatase A preparation (ASA) was also concentrated by ultrafiltration. Enzymes were stored at 4°C for months without loss of activity.

2.4. Determination of arylsulfatase activity

Arylsulfatase activity was determined with artificial substrate, 2-hydroxy-5-nitrocatechol sulfate (NCS), according to the method of Roy [23] as modified by Bleszynski [11]. Briefly, to 0.3 ml of 5 m*M* substrate solution (pH 5.6) 0.1 ml of the enzyme preparation was added. The reaction was carried out at 37°C for 10 or 20 min and then was terminated by adding 2 ml of 10% NaOH. The amount of reaction product was determined spectrophotometrically at 512 nm. The unit of activity is defined as the amount of enzyme which yields 1 μmol of 4-nitrocatechol per min in assay conditions.

2.5. Determination of sulfohydrolase activity towards UDP-GalNAc-4-S

Enzymatic reactions were carried out in Eppendorf microcentrifuge tubes in 37°C for 20 min. Typically, the reaction mixture contained 50 m*M* Tris–acetic acid, pH 5.0, 0.5 m*M* UDP-GalNAc-4-S (final concentrations) and an appropriate amount of the enzyme preparation in a total volume of 120 μl. Reaction was initiated by addition of enzyme preparation and terminated by heating in a boiling water bath for 1 min. The unit of activity is defined as the amount of enzyme desulfating 1 nmol of UDP-GalNAc-4-S per min in the reaction conditions. In the case of reaction time course studies the reaction mixture (total volume 240 μl) was incubated at 37°C and every 15 min an aliquot of 30 μl has been taken up and immediately chromatographed.

Quantitative and qualitative analyses of the reaction mixtures were performed using a HPLC system (LKB, Bromma, Sweden) consisting of two 2150 pumps, 2152 HPLC controller, diode array 2140 UV rapid spectral detector, 7125 Rheodyne sample injector (20 μl sample loop), Spherisorb

ODS-2 column (250×4.6 mm) equipped with a 10 mm guard column and precolumn filter (2 μm). Two alternative elution modes were used: (1) Gradient elution: buffer A: 20 m*M* ammonium acetate, 8 m*M* tetrabutylammonium hydroxide, acetic acid to pH 5.1; buffer B: buffer A–methanol (65:35, v/v). Gradient program: 0–5 min 0% B, 5–10 min 0–100% B (linear gradient), 10–25 min 100% B, 25–25.1 min 100–0% B (linear gradient). Each sample injection was preceded by the column reequilibration with buffer A for 10 min. In order to stabilize the retention times of eluted compounds the first two gradient runs in the sequence were performed without any sample injection. (2) The isocratic elution was carried out using buffer B. In both elution modes flow-rate was 1 ml/min. Eluents were always degassed by vacuum filtration through 0.22 μm PVDF membrane. Data acquisition and analysis were performed using either LKB Wavescan EG software (for UV spectral analysis and determination of the purity of eluted compounds) or Knauer 2.21 HPLC software (for quantitative analysis of chromatograms acquisited at 260 nm). The gradient chromatograms presented here were subjected to base line drift compensation and suppression of artifacts arising in the course of reversed-phase ion-pairing gradient HPLC.

2.6. Protein determination

Protein concentration in crude preparations was assayed by the standard biuret method and in the purified enzyme preparations by the BCA method [27] using crystalline BSA as a standard.

3. Results

3.1. Isolation of UDP-GalNAc-4-S

The procedure of UDP-GalNAc-4-S purification described by Donovan et al. [26] for fresh egg white was adapted here to isthmus tissue extract. This compound is predominant in the nucleotide pool of the tissue (Fig. 1). The purified nucleotide preparation was chromatographically homogeneous as confirmed by gradient HPLC and spectral analysis of the single peak eluted. UDP-GalNAc-4-S constitutes

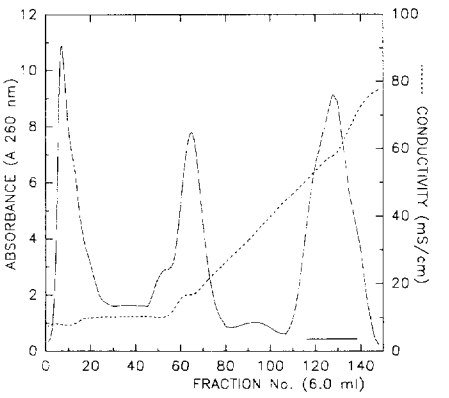

Fig. 1. Separation of hen oviduct sugar nucleotides on Dowex AG 1×10 (formate) column. The nucleotides were eluted with 0.01–1.0 *M* ammonium formate concentration gradient (– – –) as described in Section 2. UDP-GalNAc-4-S was eleuted as a last peak and pooled fractions were marked with horizontal line.

more than 98% of UV-absorbing material in the final preparation. Chemical analysis showed an equimolar ratio of N-acetylhexosamine to sulfate. The yield of the pure nucleotide estimated spectrophotometrically by using a molar absorption coefficient of 10 000 was 31 μmol per 100 g of wet tissue.

3.2. Purification of arylsulfatases B1 and A

The bovine liver protein extract obtained after dissolving the protein precipitated with acetone (20–65%, v/v) contains the comparable activities of both arylsulfatases, A and B. During chromatography on DEAE-cellulose column, arylsulfatase B1 (ASB1) activity was completely resolved from arylsulfatase B2 (ASB2) and arylsulfatase A (ASA) activities (Fig. 2). Chromatography of ASB1 on Blue-Sepharose and ConA-Sepharose columns resulted in partially purified enzyme preparation with specific activity 2727 mU/mg of protein. ASA was purified in two further steps: ConA-Sepharose and Phenyl-Sepharose and the final specific activity of the ASA preparation was 4517 mU/mg of protein. This procedure yielded highly purified, although not electrophoretically homogenous, arylsulfatase A and B1. The one part of this ASB1 preparation was finally purified by hydrophobic interaction chromatography on Phenyl-Sepha-

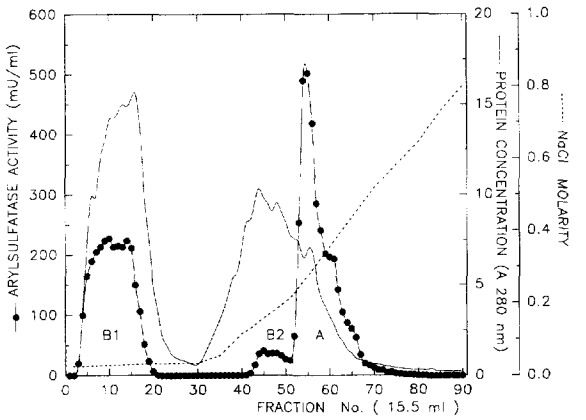

Fig. 2. Chromatographic separation of bovine liver lysosomal arylsulfatases on DEAE-cellulose column. Enzyme preparation after acetone fractionation step (110 ml containing 121 units of activity and 6.1 g of protein) was applied to the column (5.5×12 cm) equilibrated with starting buffer (20 m*M* Tris–HCl, pH 7.2).

rose and after ultrafiltration its specific activity was 11 428 mU/mg of protein.

This ASB1 preparation was devoid of other nucleotide hydrolysing enzyme activities and on 12.5% SDS-PAGE gel electropherograms, stained with Coomassie Blue R-250 the major protein band of 54 kDa and very few fairly visible additional bands were present. A summary of the purification procedure is shown in Table 1.

3.3. Enzymatic hydrolysis of UDP-GalNAc-4-S and determination of the reaction products

In preliminary experiments we established optimal reaction conditions as pH 5.0, substrate concentration of 0.5 m*M* and 30 min incubation time at 37°C. It has been shown using HPLC with isocratic elution that UDP-GalNAc-4-S is hydrolyzed by arylsulfatases B but not by arylsulfatase A or heat-inactivated ASB. The reaction product (t_R =4,13 min) can be rapidly separated from the substrate (t_R=6.92 min) (Fig. 3).

Using gradient elution we have found that more than one reaction product is detected when the substrate (t_R=18.45 min) is hydrolyzed by arylsulfatase B1 purified up to the ConA-Sepharose chromatography step. Besides the product of desulfation (UDP-GalNAc, t_R=15.50 min) three small peaks of other compounds with the characteristic uridine spectrum were resolved (Fig. 4a). The first peak (t_R=5.72 min) has not been identified yet and the next two peaks with t_R=7.32 min and 12.75 min were identified as uridine and uridine 5′-monophosphate, respectively (Fig. 4a).

The same three peaks of uridine derivatives have

Table 1
Purification of arylsulfatase B1 from 500 g of bovine liver

Step of purification	Total protein (mg)	Total activity (milliunits, mU)	Specific activity (mU/mg of protein)	Yield (%)	Purification factor
Buffer extract	26 950.0	258 720.0[a]	9.6	100	1×
Acetone fractionation	13 400.0	245 220.0[a]	18.3	95	2×
DEAE-cellulose chromatography	2440.3	101 748.0	41.7	39	5×
Blue-Sepharose chromatography	177.7	77 299.5	435.0	30	55×
ConA-Sepharose chromatography	13.7	37 359.9	2727.0	14.4	345×
Phenyl-Sepharose chromatography	2.0	22 856.0	11 428.0	4.4	1447×

[a] Sum of arylsulfatase A (ASA) and arylsulfatases B (ASB) activities.

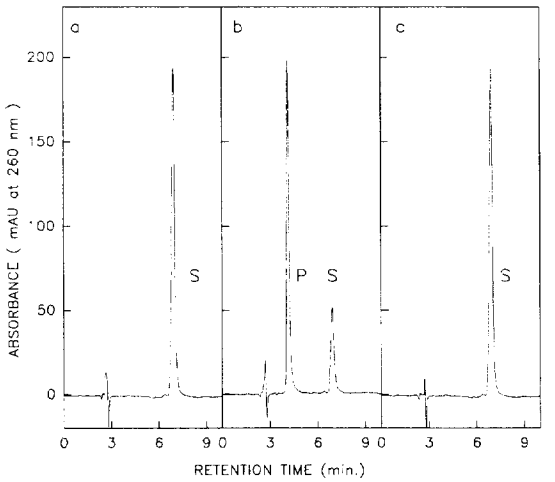

Fig. 3. Isocratic HPLC separations of incubation mixtures on Spherisorb ODS 2 column. Substrate (UDP-GalNAc-4-S) was incubated with (a) heat-inactivated arylsulfatase B1 (control), (b) arylsulfatase B1 and (c) arylsulfatase A. S – substrate (UDP-GalNAc-4-S), P – desulfation product (UDP-GalNAc).

appeared when the desulfation product (UDP-GalNAc), purified by gradient HPLC, was incubated with the above mentioned ASB1 preparation (Fig. 5).

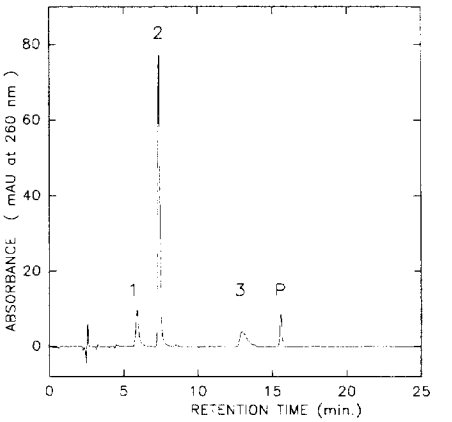

Fig. 5. Gradient HPLC separation of the products resulting from the incubation of UDP-GalNAc (obtained after desulfation of UDP-GalNAc-4-S) with partially purified arylsulfatase B1 (after ConA-Sepharose step). P – UDP-GalNAc, 1 – unidentified product, 2 – uridine, 3 – UMP.

The more purified ASB1 (after the Phenyl-Sepharose step) generates only a single reaction product, UDP-GalNAc (Fig. 4b) when assayed at optimal pH (5.0). Incubation of the substrate with the same enzyme preparation at lower pH (3.5) results in the appearance of an additional small peak of UMP as can be seen using gradient elution (Fig. 6a). This

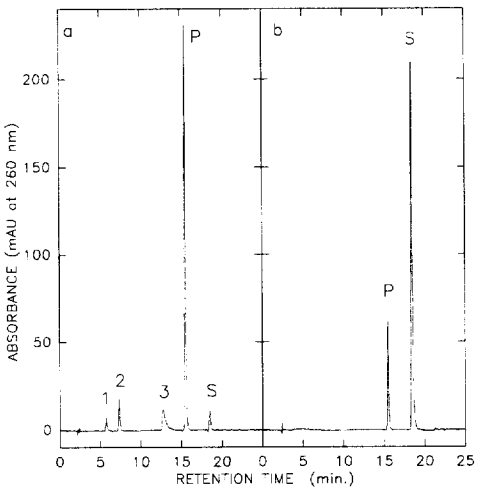

Fig. 4. Gradient HPLC separation of reaction mixtures resulting from the incubation of UDP-GalNAc-4-S with (a) partially purified arylsulfatase B1 (after ConA-Sepharose step) (b) purified arylsulfatase B1 (after Phenyl-Sepharose step). S – substrate, P – desulfation product (UDP-GalNAc), 1 – unidentified product, 2 – uridine, 3 – UMP.

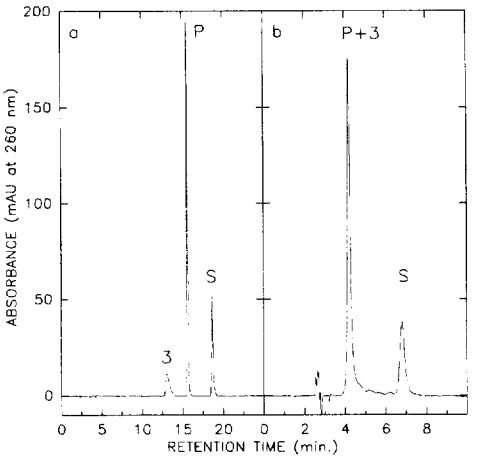

Fig. 6. Hydrolysis of UDP-GalNAc-4-S (S) by bovine arylsulfatase B1 (after Phenyl-Sepharose step) carried out at pH 3.5. (a) Besides UDP-GalNAc (P), UMP (3) an additional reaction product can be observed using gradient HPLC (see also Figs. 4 and 5). (b) Both reaction products coeluted when isocratic HPLC was applied.

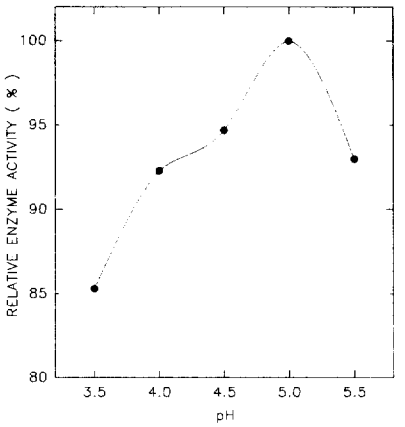

Fig. 7. pH–activity curve for bovine arylsulfatase B1 determined with UDP-GalNAc-4-S as a substrate. Desulfation product was quantitated by gradient HPLC.

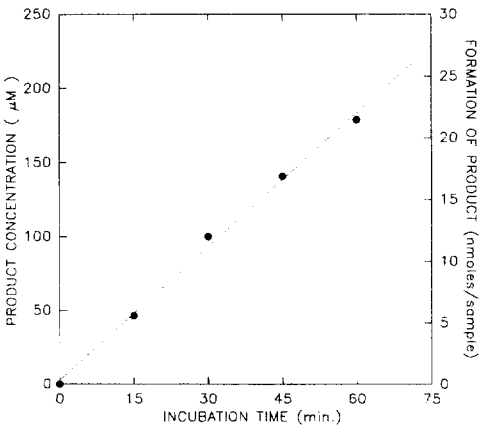

Fig. 9. Linearity of enzymatic desulfation of UDP-GalNAc-4-S by bovine arylsulfatase B1 measured up to 60 min with enzyme concentration up to 0.3 units per sample in standard reaction conditions (0.5 m*M* UDP-GalNAc-4-S, pH 5.0, 120 μl total sample volume).

compound could not be separated from the true reaction product when isocratic elution was applied (Fig. 6b).

3.4. Kinetic properties of bovine (arylsulfatase B)

At the 0.5 m*M* UDP-N-acetylgalactosamine-4-sulfate concentration, the pH optimum for the purified ASB was 5.0, as estimated on the basis of the amount of reaction product formed (Fig. 7). The reaction equilibrium is shifted toward hydrolysis and at the substrate concentration up to 300 μ*M* – at which the enzyme is saturated – over 80% of the substrate can be converted into the product (Fig. 8). At higher substrate concentration (over 500 μ*M*) we observed enzyme inhibition which was almost the total at 5 m*M* concentration. The reaction was linear with time up to 60 min (Fig. 9) and with the enzyme concentration up to 0.3 units per sample. At higher enzyme concentration the reaction was linear until about 40% of the substrate was consumed. The K_m value for bovine ASB1 was 85 μ*M* at pH 5.0 (Fig. 10).

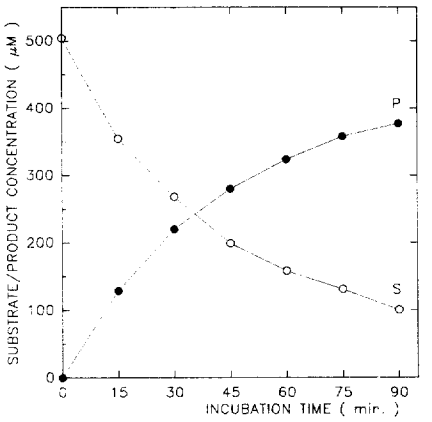

Fig. 8. Time course of the desulfation reaction of UDP-GalNAc-4-S catalyzed by bovine arylsulfatase B1 in a standard reaction conditions as expressed by means of UDP-GalNAc formation (P) and substrate decrement (S).

4. Discussion

Artificial colorimetric or fluorogenic substrates are commonly used for arylsulfatase activity assay [9]. Two groups of these enzymes, namely arylsulfatase A (ASA) and arylsulfatase B (ASB), playing different physiological roles in mammals [1] can hydrolyze these artificial substrates with similar rate and specificity [9].

For diagnostic purposes, arylsulfatase A or B activities in human tissue extracts or body fluids were differentiated by using selective inhibitors [7,8,28] or different incubation temperatures. However, these methods do not permit sufficient differentiation of both enzymes in other mammals [6] as well

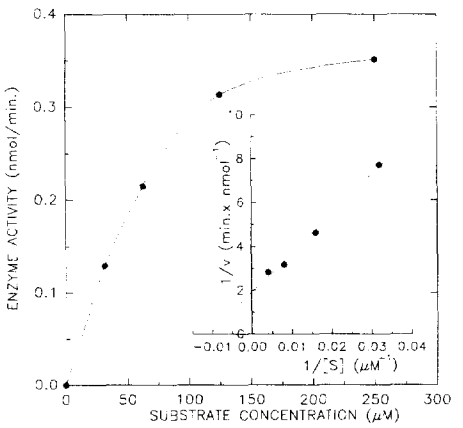

Fig. 10. Substrate concentration–activity curve for bovine arylsulfatase B1 with UDP-GalNAc-4-S as a substrate. Inset: Lineweaver–Burk double reciprocal plot. The assay was performed after incubation of 0.3 enzyme unit with increasing substrate concentration (pH 5.0) for 20 min at 37°C. The amount of product was determined after gradient HPLC separation.

as in lower vertebrates and invertebrates (unpublished results). In these cases chromatographic separation of both enzyme activities is needed and resolution on DEAE-cellulose is most commonly used. Generally, it is considered that unadsorbed or weakly bound enzymes are ASB's and stronger adsorbed arylsulfatase activity belong to ASA's. Nevertheless, during our comparative studies on phylogenetic differentiation of arylsulfatases we have found these criteria far insufficient for appropriate classification of these enzymes. It is obvious that for such classification the assay of specific arylsulfatase activity towards natural substrates is necessary. The ASB activity determination with natural substrates is rather difficult. In general, it is based on the separation and quantitation of $^{35}SO_4^{2-}$ liberated from terminal, nonreducing N-acetylgalactosamine-4-sulfate residue present in oligosaccharides derived from chondroitin-4-sulfate or dermatan sulfate [2,3,18,19] or in UDP-GalNAc-4-S [15,16,21]. All these substrates have been used only sparingly because none of them is commercially available, the isolation procedure is rather time-consuming and the half-life of ^{35}S is relatively short (88 days). For these reasons several methods of the sulfohydrolase activity determination based on HPLC separation and quantitation of desulfation products have been developed [22,29,30]. Fluharty et al. were the first to propose such a method for ASB activity determination using UDP-GalNAc-4-S as a substrate [22]. To overcome some technical difficulties they recommended the commercial dehydrated egg white as a convenient source of this nucleotide instead of fresh egg white [26]. In our preliminary experiments we have found that nucleotide content in dehydrated egg white we had bought was relatively low. Moreover, the supernatants obtained after centrifugation of the boiled, either dehydrated or fresh, egg white clogged the Dowex-1 column and seriously decreased the flow-rate. So, we have decided to isolate the UDP-GalNAc-4-S from hen oviduct which is the most abundant source of the sulfated sugar nucleotides [24,25]. We adapted and simplified the method of Donovan et al. for purification of this compound. The preparation is relatively simple and the compound of interest can be separated from other sugar nucleotides in one chromatographic run (Fig. 1). After the charcoal adsorption step this compound was at least 98% pure as was determined by chemical, spectral and HPLC methods, and the final yield was 31 μmol/100 g of tissue. This yield is a little higher than Strominger reported for the same material [24] and about 6-fold higher in comparison to fresh egg white preparation [26].

We have used this compound for ASB activity determination according to the method of Fluharty et al. [22]. However, we have found that the proposed chromatographic conditions result in relatively long analysis time and significant substrate peak broadening and tailing. For these reasons we have modified chromatographic conditions using a longer RP C_{18} column and changing the eluent composition to 20 m*M* ammonium acetate, 8 m*M* tetrabutylammonium hydroxide, pH 5.1–methanol (65:35, v/v). Significantly shorter run times with excellent substrate and product peaks separation were achieved. Both compounds were eluted as sharp and symmetrical peaks thus improving the quantitative analysis (Fig. 3). The substrate is selectively hydrolyzed by bovine ASB but not ASA, similar to the case of human [16] and hen [15] arylsulfatases. However, when using an impure preparation this sulfated sugar nucleotide can be catabolized by other nucleotide metabolizing enzymes, which interfere with the ASB assay [21]. For this reason we attempted to use this substrate for

ASB determination in enzyme preparations of different purity using a high resolution gradient HPLC method as described in Section 2. In the case of partially purified ASB1 (after ConA-Sepharose step) we have observed three smaller uridine derivative peaks in addition to the essential desulfation product (Fig. 4a). Two of three peaks have been identified as uridine (t_R=7.32 min) and UMP (t_R=12.75 min) and it indicates the presence of phosphodiesterase and phosphomonoesterase in ASB preparation. The sequence of reactions leading to these products is not elucidated but we have demonstrated that these additional compounds can also be formed from UDP-GalNAc (Fig. 5). Contaminating enzyme activities can be removed by the chromatography on Phenyl-Sepharose and the resulting bovine ASB1 preparation catalyzed only the desulfation reaction (Fig. 4b). Surprisingly, even this ASB1 preparation produces UMP (peak 3, t_R=12.75 min) when the reaction is carried out at a pH lower (Fig. 6) than the optimum (Fig. 7). Although it is possible that our enzyme preparation still contains a trace amount of another nucleotide metabolizing enzyme, it can not be excluded that ASB1 possesses an additional activity, as was observed in the case of bovine ASA [31]. This problem is currently under study.

The most important feature of the described method is its sensitivity and reliability. The detection limit is 0.1 nmol of the product and the standard error of determination for a repeatedly injected sample does not exceed 3%. This makes this method especially useful for kinetic study as was shown for bovine ASB1. The kinetic properties of this enzyme determined by the described method, with a pH optimum of 5.0, optimal substrate concentration about 0.5 m*M* and K_m=85 μ*M* (Fig. 7 and Fig. 10), closely resemble that of hen oviduct and rat liver and kidney ASB's [15,21], but differ from human ASB which exhibits lower pH optimum (3.5) and very high affinity to the substrate with K_m=3.2 μ*M* [22].

In the routine HPLC method of ASB activity determination, in order to insure linearity of the reaction, 0.5 m*M* substrate concentration and incubation time up to 60 min is recommended, but the enzyme activity in a sample must be so adjusted that not more than 40% of the substrate be hydrolyzed (Fig. 9).

Although the isocratic HPLC method should not be used for ASB activity determination in crude preparations because of poor resolution of different reaction products, this method may be very useful for work with purified arylsulfatase B types from different sources. The gradient elution method permits the selective detection of specific desulfation product and therefore should be applied for partially purified ASB's. Preliminary research devoted to arylsulfatases phylogeny confirmed the usefulness of both methods to distinguish ASA from ASB in lower vertebrates [14].

Acknowledgments

This work was supported in part by Grant 420B/94 from Copernicus University.

References

[1] E.F. Neufeld, Annu. Rev. Biochem. 60 (1991) 257–280.
[2] J.F. O'Brien, M. Cantz, J. Spranger, Biochim. Biophys. Res. Commun. 60 (1974) 1170–1177.
[3] J. Gniot-Szulzycka, P.V. Donelly, FEBS Lett. 65 (1976) 63–68.
[4] S. D Gorham, M. Cantz, Hoppe-Seyler's Z. Physiol. Chem. 359 (1978) 1811–1814.
[5] A.L. Fluharty, in: J.W. Callahan, J.W. Lowden (Eds.), Lysosomes and Lysosomal Storage Diseases, Raven Press, New York, 1981, pp. 249–261.
[6] M. Worwood, K.S. Dodgson, G.E.R. Hook, F.A. Rose, Biochem. J. 134 (1973) 183–190.
[7] E. Shapira, H.L. Nadler, Clin. Chim. Acta 65 (1975) 1–4.
[8] R. Mercelis, A.F. Van Elsen, J.G. Leroy, Clin. Chim. Acta 93 (1979) 85–92.
[9] A.B. Roy, Methods Enzymol. 143 (1987) 207–217.
[10] A.J. Leznicki, M. Rozanska, Acta Biochim. Polon. 38 (1991) 151–156.
[11] W. Bleszynski, A.J. Leznicki, Enzymologia 33 (1967) 373–389.
[12] A.J. Leznicki, Acta Univ. N. Copernici (Biol.) 31 (1988) 65–84.
[13] W.L. Daniel, L. Leonard, T. Carmody, Comp. Biochem. Physiol. 87B (1987) 385–390.
[14] A.J. Leznicki, K. Bialkowski, Proc. 30th Meeting Pol. Biochem. Soc. (Szczecin), 1994, p. 232.
[15] M. Tsuji, M. Hamano, Y. Nakanishi, K. Ishihara, S. Suzuki, J. Biol. Chem. 249 (1974) 879–885.
[16] A.L. Fluharty, R.L. Stevens, D. Fung, S. Peak, H. Kihara, Biochem. Biophys. Res. Commun. 64 (1975) 955–962.
[17] A. Poulos, K. Beckman, Clin. Chim. Acta 89 (1978) 417–420.

[18] R.L. Stevens, A.L. Fluharty, J.F. Wall, D.A. Maple, H. Kihara, Carbohydr. Res. 76 (1979) 295–302.
[19] H. Habuchi, M. Tsuji, Y. Nakanishi, S. Suzuki, J. Biol. Chem. 254 (1979) 7570–7578.
[20] G. Dubois, B. Zalc, F. Le Saux, N. Baumann, Anal. Biochem. 102 (1980) 313–317.
[21] M. Tsuji, Y. Nakanishi, H. Habuchi, K. Ishihara, S. Suzuki, Biochim. Biophys. Acta 612 (1980) 373–383.
[22] A.L. Fluharty, J.A. Glick, G.F. Samaan, H. Kihara, Anal. Biochem. 121 (1982) 310–314.
[23] A.B. Roy, Biochem. J. 68 (1958) 519–528.
[24] J.L. Strominger, J. Biol. Chem. 237 (1962) 1388–1392.
[25] Y. Nakanishi, H. Sonohara, S. Suzuki, J. Biol. Chem. 245 (1970) 6046–6051.
[26] J.W. Donovan, J.G. Davis, L.U. Park, Arch. Biochem. Biophys. 122 (1967) 17–23.
[27] P.K. Smith, R.J. Krohn, G.T. Hermanson, A.K. Malia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[28] H. Baum, K.S. Dodgson, B. Spencer, Clin. Chim. Acta 4 (1959) 453–455.
[29] M. Ohsaki, T. Matsumoto, N. Sakura, K. Ueda, Clin. Chim. Acta 215 (1993) 165–171.
[30] A. Shilatifard, R.D. Cummings, Glycobiology 5 (1995) 291–297.
[31] T. Uchida, F. Egami, A.B. Roy, Biochim. Biophys. Acta 657 (1981) 356–363.