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Bovine Testicular Heparin Sulfamidase

Properties and Partial Purification

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Heparin sulfamidase was purified 2200-fold in 7% yield from bovine testis by a procedure which consisted of homogenization of the tissue in the presence of Triton X-100, ammonium sulfate fractionation, chromatography on concanavalin A-Sepharose, chromatography on heparin-Sepharose, and chromatofocusing. As shown by SDS-PAGE, the most highly purified preparation contained one major component, which had a mobility corresponding to a molecular weight of 55 000. Chromatography of the active enzyme on Sephacryl S-300 indicated a molecular weight of 100 000, suggesting that the native enzyme consisted of two subunits. K_M for the purified enzyme was 1.6 μM, assayed with [*N*-^{35}S]heparin as a substrate; V_{max} was 4.4 pmol of sulfate released per min per μg of protein. A pH of 6.7 was estimated from the elution profile on chromatofocusing and was corroborated by cold focusing electrophoresis. The enzyme was optimally active between pH 4.8 and 5.1. Maximum activity was observed in the presence of 0.175 M NaCl, while lower activities were observed upon addition of other salts.

Heparin sulfamidase (EC 3.10.1.1), is a lysosomal acid hydrolase which acts upon the *N*-sulfate groups of non-reducing terminal glucosamine residues in heparin and heparan sulfate and is essential for the normal catabolism of these polysaccharides [1]. A deficiency in this enzyme results in the Sanfilippo syndrome (type A), a human genetic disorder which is characterized clinically by severe progressive psychomotor retardation and relatively mild skeletal anomalies [2-4]. Biochemically, the disease is manifested by accumulation of partially degraded heparan sulfate in the tissues and excretion of large quantities of this material in the urine. The existence of sulfamidase in mammalian tissues was first shown by Lloyd *et al.* [5] and Lemaire *et al.* [6] who detected inorganic [^{35}S]sulfate in the urine of rats that had been injected with [*N*-^{35}S]heparin. Lloyd *et al.* [7] also demonstrated that a cell free extract from rat spleen released inorganic [^{35}S]sulfate from [*N*-^{35}S]heparin. These results were confirmed by Dietrich [8]

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who reported the presence of sulfamidase activity in ileum, lung and lymphoid tissue of rats. Sulfamidase was shown to be a lysosomal enzyme by Friedman and Arsenis [9, 10] who also demonstrated that it was distinct from arylsulfatase A and B. Kresse and Neufeld [11] purified a factor from normal human urine which was capable of correcting the metabolic deficiency in Sanfilippo A fribroblasts and this factor was subsequently shown to be sulfamidase [12].

More recently, sulfamidase was purified extensively from human placenta by Paschke and Kresse [13], and the isolation of a homogeneous preparation, obtained from human liver after 20 000-fold purification, was reported by Freeman, Hopwood and coworkers [14, 15]. In the present communication, we describe the partial purification of sulfamidase from bovine testis and studies of some properties of the enzyme from this source. A preliminary report has appeared [16] (see also [17]).

Experimental Procedures

Reagents were purchased as follows: *p*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-α-L-fucoside, *p*-nitrocatechol sulfate, concanavalin A, methyl-α-D-mannoside, porcine intestinal mucosa heparin (158 units/mg), Triton X-100, cetylpyridinium chloride, and bovine serum albumin from Sigma Chemical Company (St. Louis, MO, USA); *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide from Aldrich Chemical Company (Milwaukee, WI, USA); Bio-Gel P-2, electrophoresis supplies, and molecular weight standards for gel chromatography and SDS-PAGE from Bio-Rad Laboratories (Richmond, CA, USA); Silane A174, Sepharose 4B and 6B, AH-Sepharose, Sephacryl S-300, Pharmalyte (pH 3.0 to 10), supplies for chromatofocusing, Polybuffer exchanger 94, Polybuffer 74, and Polybuffer 96 from Pharmacia Fine Chemicals (Uppsala, Sweden); enzyme grade ammonium sulfate from Schwarz/Mann (Cleveland, OH, USA); bovine testes from Pel-Freez Biologicals, Inc. (Rogers, AR, USA); ScintiVerse I from Fisher Scientific (Norcross, GA, USA); and [*N*-^{35}S]heparin (lot no. F50371), with a specific activity of 17.5 mCi/g, from Amersham Corp. (Arlington Heights, IL, USA). Prior to use, the radiolabeled heparin was separated from any contaminating inorganic sulfate by chromatography on a column (1 × 122 cm) of Bio-Gel P-2 in 0.2 M NaCl. Nitrous acid treatment at pH 1.5 and 4.0 [18], followed by gel chromatography as indicated, confirmed that the ^{35}S-label was located exclusively in the *N*-sulfate groups.

Concanavalin A was coupled to Sepharose as described [19] using the method of Nishikawa and Bailon [20] for activation of the gel. Heparin-Sepharose was prepared by coupling heparin to AH-Sepharose by the method of Cuatrecasas [21].

Buffers

The following buffers were employed: (A) 0.1 M sodium citrate, pH 6.0, 0.1% Triton X-100, and 0.01% sodium azide; (B) 0.1 M sodium citrate, pH 6.0, and 0.01% sodium azide; (C) 0.05 M sodium acetate, pH 5.0; (D) 0.025 M Tris-acetate, pH 8.3; (E) 70% Polybuffer 74 and 30% Polybuffer 96, pH 5.0; and (F) 0.05 M sodium acetate, pH 4.5, 0.15 M NaCl, and 0.01% sodium azide.

Enzyme Assays

Heparin sulfamidase activity was determined by a modification of the method of Thompson [22], with incubation times of 16 to 120 min. Reaction mixtures contained the following components in a total volume of 0.5 ml: enzyme (20 μl; 98 ng to 20 μg of protein); buffer F (470 μl); and [*N*-^{35}S]heparin (10 μl; 10^5 cpm). After incubation at 37°C, the reaction was terminated by the addition of 100 μl of unlabeled heparin (40 mg/ml) and 200 μl of 5% cetylpyridinium chloride containing 0.1 M Na_2SO_4 and 0.2 M NaCl. The mixture was incubated at 37°C for 30 min, and the precipitate was removed by centrifugation for 5 min at 15 600 × *g* in an Eppendorf centrifuge (Model 5412). A 200 μl aliquot of the supernatant was removed, mixed with 300 μl of water and 3 ml of ScintiVerse I, and the radioactivity was measured in a Packard model 3375 liquid scintillation spectrometer. The specific activity of the sulfamidase was expressed as pmol [^{35}S]sulfate released/min/μg of protein. Specific activities were calculated on the assumption that the heparin used as a substrate had a molecular weight of 10 000 and contained one *N*-sulfate group per molecule which was susceptible to cleavage by sulfamidase.

Other acid hydrolases were measured according to the methods described by Hall *et al.* [23]. Reaction mixtures contained 100 μl of a 5 mM solution of the appropriate *p*-nitrophenyl glycoside or 50 mM *p*-nitrocatechol sulfate, 100 μl of enzyme (10-50 μg of protein), and 300 μl of the appropriate buffer. Incubation times were 5-20 min for assay of the crude testis enzyme and 18 h for the assay of contaminating hydrolases in the most highly purified sulfamidase preparation.

Analytical Methods

Protein was measured by the method of Lowry *et al.* [24] or by the method of Bradford [25]. Uronic acid content of heparin was estimated by the method of Dische [26].

Estimates of the molecular weight of sulfamidase were obtained by gel chromatography on a column (2.5 × 95 cm) of Sephacryl S-300, which was eluted with 0.1 M sodium citrate-phosphate buffer, pH 8.0, or with the same buffer containing 0.1% Triton X-100. The column was standardized with a mixture of molecular weight markers (thyroglobulin, gammaglobulin, ovalbumin, myoglobin, and vitamin B_{12}).

SDS-PAGE was performed at 5°C in a Bio-Rad tube gel apparatus according to the method of Laemmli [27]. Gels were subjected to electrophoresis for 2 h prior to sample application. After electrophoresis, proteins were visualized with Coomassie blue R-250.

Polyacrylamide isoelectric focusing gels (pH 3.5 to 10) were prepared on silanized glass plates by a modification of the method of Radola [28]. The ultrathin-layer gels (160 μm) used for cold focusing contained 5% acrylamide and 3% ampholyte and were prepared as follows. Acrylamide (2.9 g) and bisacrylamide (86 mg) were dissolved in 3.3 ml of water. A 1.1 ml aliquot of this solution was mixed with 1.1 ml of ampholyte (pH 3.5 to 10), 5.3 ml of water, 7.6 ml of 0.1% ammonium persulfate, and 10 μl of *N,N,N',N'*-tetramethylethylenediamine.

Cold focusing was performed on a Cold Focus apparatus (MRA Corp., Clearwater, FL 33515, USA) with a separation distance of 5.5 cm [29]. Samples (5 to 10 μl) were applied to the gel close to the cathode, using 1 × 3 mm tabs of Whatman No. 1 filter paper. The cathode and anode solutions were 1 M NaOH and 1 M H_3PO_4, respectively. Heat dissipation during the electrofocusing procedure was controlled by use of electronic

Table 1. Instrument settings for a typical cold focusing experiment.

Cooling (amp)	Watts	Volts	Milliamps	Time (min)
1	1.5	200	9.75	10
2	4	495	9.75	10
4	6	1350	5.25	10
6	8	1900	4.75	10
6	10	2500	4.75	4

dual Peltier devices in conjunction with a beryllium oxide plate; the maximum current (maximum cooling) that could be applied to the Peltier devices was 6 amperes. Constant power for electrofocusing was provided by a 4000 V Constant Power Supply (MRA Corp.). The instrument settings for a typical cold focusing experiment are shown in Table 1. After electrofocusing, proteins were visualized by a modification of the silver nitrate staining method [30].

Purification of the Sulfamidase

Extraction of Tissue. All procedures were conducted at 4°C unless otherwise specified. The frozen tests (7.5 kg) were trimmed of epididymis and external connective tissue, cut into pieces approximately 3 mm thick, and homogenized in a Waring blender for 90 s (3 × 30 s) at high speed in 2 vol of buffer A. A total of 4.8 kg of trimmed tissue was processed in this manner.

Ammonium Sulfate Fractionation. The homogenate was centrifuged at 10 000 × *g* for 90 min, and solid ammonium sulfate (1.24 kg) was added to the supernatant (13.5 l), resulting in 20% saturation. After stirring for 4 h, the precipitate was removed by centrifugation (10 000 × *g* for 90 min), ammonium sulfate was added to the supernatant to 40% saturation, and the mixture was stirred for 4 h. The precipitate was collected by centrifugation at 10 000 × *g* for 1 h, dissolved in one liter of buffer B, and dialyzed for 20 h with five changes of 4 l each of buffer B.

Concanavalin A-Sepharose Chromatography. The solution from the preceding step was clarified by centrifugation for 1 h at 2000 × *g*, and the supernatant (2.6 l; 79.6 g of protein) was applied to a column (4.5 × 56 cm) of concanavalin A-Sepharose, which had been equilibrated with buffer B. The column was eluted with buffer B until the absorbance of the effluent at 280 nm was less than 0.1. The column was then allowed to warm to room temperature, and the sulfamidase was eluted with buffer B containing 0.5 M methyl-α-D-mannoside [31]. Fractions containing the enzyme were combined and concentrated by filtration through an Amicon YM-10 membrane with repeated additions of buffer C.

Heparin-Sepharose Chromatography. One third of the preparation (500 ml) from the preceding step was applied to a column (7 × 16 cm) of heparin-Sepharose which had been equilibrated with buffer C. The column was washed with 3 bed volumes of buffer C, and the sulfamidase was then eluted with buffer C containing 0.2 M NaCl. Fractions

Table 2. Purificaton of heparin sulfamidase.

Procedure	Volume (l)	Protein concentration (mg/ml)	Sulfamidase activity: Total activity (units[b])	Sulfamidase activity: Specific activity[a] (units/mg)	Purification (-fold)	Yield (%)
Homogenization	13.5	33.65	117400	0.26	1.0	100
Ammonium sulfate fractionation	2.6	30.60	46800	0.59	2.3	40
Con A-Sepharose chromatography	1.4	3.43	47900	10	39	41
Heparin-Sepharose chromatography[c]	0.44	0.32	10500[c]	74	288	27
Chromatofocusing	0.01	0.49	2800	560	2187	7

[a] The specific activity was determined at a substrate concentration below saturation.
[b] Units are defined as pmol of sulfate released/min.
[c] Only a third of the sulfamidase pool from Con A-Sepharose chromatography was processed through the remaining steps.

(20 ml) were collected, and those with sulfamidase activity were combined, concentrated to approximately 5 ml on a YM-10 membrane, and dialyzed against 3 changes (500 ml each) of buffer D.

Chromatofocusing

Chromatofocusing was performed on Polybuffer Exchanger 94 as described by Conary *et al.* [32], with a buffer range of pH 8 to pH 5. The material from the previous step (5 ml) was applied to a column (1 × 27 cm) of Polybuffer Exchanger 94 which had been equilibrated with buffer D. Sulfamidase was eluted with buffer E.

Results and Discussion

Purification of Sulfamidase from Bovine Testis

The most highly purified sulfamidase preparation from bovine testis was obtained in 7% yield after 2200-fold purification and had a specific activity of 560 pmol sulfate/min/mg protein (Table 2). The purification procedure consisted of (a) homogenization in the presence of detergent, (b) ammonium sulfate fractionation, (c) chromatography on concanavalin A-Sepharose, (d) chromatography on heparin-Sepharose, and (e) chromatofocusing. Some aspects of this procedure deserve comment, as follows. In our initial experiments, sulfamidase was extracted from testis tissue by homogenization and repeated freezing and thawing; however, it was found that the presence of Triton X-100 during homogenization greatly increased the yield of soluble enzyme, and the detergent was therefore used in the large-scale preparation shown in

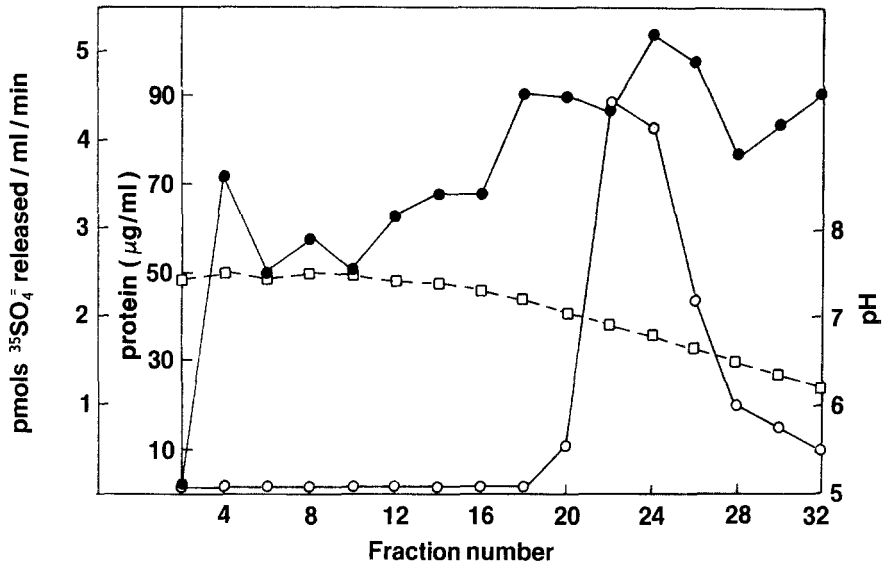

Figure 1. Chromatofocusing of heparin sulfamidase. □, pH; ●, protein concentration; ○, heparin sulfamidase activity.

Table 2. To our knowledge, the effect of detergent on the yield of soluble enzyme has not been assessed in other studies of sulfamidase. Fractionation with ammonium sulfate yielded a 2-fold purification but, at the same time, resulted in an apparent loss of 60% of the total activity. Keeping in mind that inorganic sulfate inhibits the enzyme (see below [7, 8, 10, 33]) and that some sulfate may have remained in this fraction, it is possible that its activity may have been underestimated. This interpretation seems even more likely in view of the concanavalin A-Sepharose, where some losses are usually incurred [13, 34]. This step, which provided a 17-fold purification of the testis enzyme, has also been used by others [13] and was particularly effective in the purification of sulfamidase from human liver by Mahuran *et al.* [15], who used a recycling procedure in which the eluate from the lectin column was adsorbed to Blue A Agarose.

Chromatography on heparin-Sepharose resulted in an additional 7-fold purification with a recovery of about two thirds of the applied activity. A similar step was used by Pashke and Kresse [13] in their purification of sulfamidase from human placenta. Chromatography on heparan sulfate which had been immobilized on Bio-Gel P-150 yielded two forms of the enzyme, which differed, with respect to their K_M values with heparan sulfate as a substrate. No evidence for the existence of multiple forms of the testis enzyme was found in the present study.

In the final purification step, the eluate from heparin-Sepharose was subjected to chromatofocusing on Polybuffer Exchanger 94, which yielded the elution profile shown in Fig. 1. Approximately 75% of the enzyme activity was lost in this step, but even so, the specific activity increased 7-fold to give an overall purification of 2200-fold. The enzyme was eluted in a pH range of 6.6 to 6.8; the pH indicated by this elution position was 2 pH

units above that previously reported for sulfamidase from rat spleen [10] but was in good agreement with the value of 6.8 measured by Freeman and Hopwood [14] for the human liver enzyme.

Further purification was attempted using gel chromatography on Sepharose 6B, ion-exchange chromatography on CM-cellulose, and dye ligand chromatography on Cibacron Blue-Sepharose [7, 13, 15]. We had previously used these methods successfully at earlier stages of the purification [16, 17, 35]; however, when applied to the highly purified preparation obtained after chromatofocusing, each of these procedures resulted in a substantial decrease in specific activity. These observations are in accord with the experience of others [13] who have also found that highly purified sulfamidase is labile on further manipulation. It should be noted in this context, however, that Freeman and Hopwood [14] have recently reported that highly purified enzyme from human liver is stable on storage in 15 mM dimethylglutarate, pH 6.0, containing 0.32 M NaCl, 10% glycerol, and 0.1 mM dithiothreitol.

Properties of Sulfamidase

Chromatography of partially purified sulfamidase (eluate from concanavalin A-Sepharose) on Sephacryl S-300 indicated a molecular weight of 110 000. The presence of 0.1% Triton X-100 in the eluant led to a shift in the elution positions of the sulfamidase and the protein standards towards higher effluent volumes but did not alter the molecular weight estimate.

SDS-PAGE of the enzyme preparation obtained after chromatofocusing (Table 2) yielded six major bands and several diffusely staining areas. However, a similar preparation, which had been purified on a column of previously unused heparin-Sepharose prior to chromatofocusing, gave a single band which had migrated to a position corresponding to a molecular weight of 55 000. Diffusely stained material which had migrated faster than lysozyme was also seen.

Thus, the bovine testis sulfamidase was similar in size to the enzymes from human placenta [13], human liver [14], and rat skin [33], and the presence of two subunits with M_r 55 000, suggested by SDS-PAGE, was in agreement with the results of Freeman and Hopwood [14].

The pH of 6.6-6.8 estimated in the course of purification by chromatofocusing was further corroborated by the results of cold focusing. The protein pattern seen on silver staining of enzyme purified through this step showed five discrete bands and some more diffusely staining bands, which were all in the range of pH 6.5 to 7.0, as indicated by the position of human hemoglobin (pH 6.8).

The purified sulfamidase was also examined for the presence of other lysosomal enzymes. After prolonged incubation (18 h), low levels of activity of the following enzymes were detected: β-D-galactosidase, β-hexosaminidase, β-D-glucuronidase, *N*-acetyl-α-D-glucosaminidase, α-L-fucosidase, and arylsulfatase A. The highest residual activity was observed for β-D-glucuronidase, but even for this enzyme the remaining activity amounted to only 0.003% of the initial total activity in the homogenate.

Kinetic studies of the most highly purified sulfamidase preparation showed that product formation was proportional to enzyme concentration up to 98 ng/ml and linear

with time up to 30 min. The K_M of the sulfamidase in buffer F was 1.6 μM [*N*-^{35}S]heparin, and V_{max} was 4.4 pmol of sulfate released per min per μg of protein. These values were obtained with a nonlinear regression program (SAS Institute, Inc., Gary, NC, USA). Enzymatic activity was optimal between pH 4.8 and 5.1. The enzyme was inactive in the absence of sodium chloride, and optimal activity was observed in the presence of this salt at a concentration of 175 mM. Other salts tested included NaF (150 mM) and LiCl (150 mM), which reduced the activity of the sulfamidase to 8 and 21%, respectively, of the value observed in the presence of 150 mM NaCl. The enzyme retained 48% of its activity in the presence of 75 mM magnesium chloride, but 93% inhibition was observed at a concentration of 150 mM. Sodium sulfate inhibited the reaction by 50% at a concentration of 0.25 mM. The nature of the inhibition by sulfate was not determined, but other investigators, who have observed the same phenomenon, have reported that it is noncompetitive [10].

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