Purification of a 75 kDa protein from the organelle matrix of human neutrophils and identification as *N*-acetylglucosamine-6-sulphatase

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A 75 kDa protein was purified to homogeneity from granule extracts of normal human granulocytes using Sephadex G-75 chromatography, Mono-S cation exchange chromatography and chromatofocusing. The protein consisted of one chain with a molecular mass of 75 kDa, as determined by SDS/PAGE. Tryptic peptide analysis by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS and sequence analysis by MS/MS identified the protein to be *N*-acetylglucosamine-6-sulphatase (EC 3.1.6.14). The identity of the protein was confirmed by demonstrating enzymatic activity towards the substrate *N*-acetylglucosamine 6-sulphate. The enzyme was active over a broad pH range with an optimum of pH 7.0, and showed a K_m value of 13.0 mM and a V_{max} value of $\sim 1.8 \mu\text{M}/\text{min}$ per mg.

The enzyme also showed *O*-desulphation activity towards heparan sulphate-derived saccharides. Subcellular fractionation of neutrophil organelles showed the presence of enzymatic activity mainly in the same fractions as primary granules. Furthermore, PMA treatment of the neutrophils induced release of the enzyme, indicating its matrix protein nature. The presence of *N*-acetylglucosamine-6-sulphatase in human neutrophils implies that neutrophils may play a role in the modulation of cell surface molecules and extracellular matrix by *O*-desulphation.

Key words: granule, *N*-acetylglucosamine-6-sulphatase, neutrophil, *O*-desulphation, organelle matrix, release.

INTRODUCTION

The neutrophil is the body's first line of defence against invading micro-organisms, and plays an important role both in innate immunity and in inflammatory reactions in human disease [1]. When the body encounters an inflammatory stimulus, circulating neutrophils leave the bloodstream and migrate into the tissues, where they engulf and destroy invading micro-organisms. Apart from secretory vesicles, neutrophils contain azurophil, specific (secondary) and gelatinase-containing granules [2] that are formed in the bone marrow at subsequent stages of neutrophil maturation [3]. These granules and the secretory vesicles contain different matrix and membrane proteins. It is believed that the secretory vesicles are mobilized first upon stimulation, followed by the gelatinase-containing, the secondary and the azurophil granules [4,5]. During release, the secretory vesicles incorporate membrane receptors and adhesion molecules into the plasma membrane, which allows the neutrophil to adhere to the endothelium [6]. Exocytosis of gelatinase-containing granules occurs subsequently, followed by that of secondary granules, which causes the release of matrix-degrading proteins, probably facilitating diapedesis and migration of neutrophil into the tissues [7]. Upon phagocytosis, the azurophil granules fuse with the phagosomes. This process causes the release of proteolytic and bactericidal factors into the phagolysosome, where the invading micro-organism is killed and digested [1].

The identification and characterization of unrecognized granule proteins and the determination of their subcellular localization in human neutrophils is an important approach to studying the functions of neutrophils. During the investigation of granule proteins, we found a 75 kDa protein that had not been described in

human neutrophils. To increase our knowledge of this protein we have purified and characterized it. The biochemical and enzymatic data indicate that this 75 kDa protein is an *N*-acetylglucosamine-6-sulphatase (EC 3.1.6.14).

A human *N*-acetylglucosamine-6-sulphatase has been purified previously from urine and liver [8,9]. The enzyme cleaves the *O*-linked sulphate group at the 6-position of non-reducing-end glucosamine residues of heparin, heparan sulphate and keratan sulphate [10–12]. *N*-Acetylglucosamine 6-sulphate has been reported to occur in many proteoglycans [13,14]. Various studies suggest that sulphate residues in proteoglycans may play important roles in mediating the interaction of the sulphated proteoglycans with specific proteins and a variety of microbial agents [15–18].

A deficiency in the activity of *N*-acetylglucosamine-6-sulphatase has been reported to cause the accumulation of partially degraded heparan sulphate in lysosomes and the increased excretion in urine of the monosaccharide *N*-acetylglucosamine 6-sulphate. The lysosomal accumulation of heparan sulphate results in organelle, cell and tissue distortion, leading ultimately to the lysosomal storage disorder mucopolysaccharidosis type IIID, or Sanfilippo D syndrome [10].

Many human tissues, such as liver, spleen, heart, kidney, cerebrum, placenta, fibroblasts and leucocytes [8,19], have been shown to display sulphatase activities. The urinary enzyme consists of a single polypeptide with a molecular mass of 97 kDa. The pI value of the urinary enzyme was reported to vary between 5.4 and 8.3 [8]. The human-liver-derived enzyme was found to occur in three forms. The major form is a single polypeptide of 78 kDa with a pI greater than 9.5, which is believed to be cleaved into two minor forms with molecular sizes of 48 and 32 kDa respectively

Abbreviations used: DMAB, *p*-dimethylaminobenzaldehyde; HEK, human embryonic kidney; HNL, human neutrophil lipocalin; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight.

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[9]. The cDNA clone encoding the complete amino acid sequence for the entire human *N*-acetylglucosamine-6-sulphatase has also been reported [20,21]. So far, however, the enzyme has not been described in human neutrophils, and its function in neutrophils remains to be established. In the present paper, therefore, we describe the purification and characterization of the enzyme from normal human granulocytes, as well as its localization in the matrix of the organelles of human neutrophils.

EXPERIMENTAL

Preparation of granule proteins

Granules were isolated from buffy coats of normal human blood by a modification of a method described previously [22]. The buffy coats (approx. 5 litres) were mixed with an equal volume of 2% Dextran T-500 in Dulbecco's PBS without calcium and magnesium. The granulocyte-rich plasma was collected after sedimentation of the erythrocytes for 1 h at room temperature. The granulocytes were washed twice in PBS and once in 0.34 M sucrose by centrifugation at 400 *g* for 10 min. The granulocyte pellet was resuspended in 5 vol. of 0.34 M sucrose. Isolated cells were then disrupted by nitrogen cavitation. The cell suspension was mixed with an equal volume of 0.34 M sucrose and the cells were pressurized at 4 °C for 30 min under nitrogen at 5.2 MPa (52 bar) with constant stirring in a nitrogen bomb (Parr Instrument Company, Moline, IL, U.S.A.). The cavitate was then collected into an equal volume of 0.34 M sucrose/0.3 M NaCl and centrifuged for 20 min at 450 *g* at 4 °C. The supernatant was centrifuged for 20 min at 10 000 *g* at 4 °C to sediment the granules. After one cycle of freezing and thawing, the granules were extracted with 5 vol. of 50 mM acetic acid for 1 h at 4 °C. An equal volume of 0.4 M sodium acetate, pH 4.0, was added and the extraction procedure was continued with magnetic stirring for 4 h at 4 °C. The granule extract was then concentrated to approx. 5 ml using YM-2 filter (Amicon Corp., Lexington, KY, U.S.A.).

Chromatographic procedures

Gel filtration was performed on a Sephadex G-75 Superfine column (2.5 cm × 90 cm) (Amersham Biosciences, Uppsala, Sweden) equilibrated with 0.2 M sodium acetate, pH 4.5. Ion-exchange chromatography was performed using the FPLC system (Amersham Biosciences) on a strong cationic exchanger Mono-S prepacked column equilibrated with 0.1 M sodium acetate, pH 4.0. The bound proteins were eluted with a linear gradient from 0 to 1.0 M NaCl in 0.1 M sodium acetate, pH 4.0. The proteins eluted in the first peak were applied to the same column equilibrated with 0.02 M sodium acetate, pH 5.0. The bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 0.02 M sodium acetate, pH 5.0. Chromatofocusing was performed on a Mono-P prepacked column equilibrated with 0.025 M diethanolamine, pH 9.5. The bound proteins were eluted with Polybuffer 74, pH 4.0.

The proteinase inhibitors PMSF (100 mg/l) and soybean trypsin inhibitor (100 mg/l) were added to all buffers from the cell disruption step to the first ion-exchange chromatography step. Proteins in the chromatograms were measured by their absorbance at 280 nm. Ultrafiltration of pooled fractions was performed using YM-10 filters. Buffer exchange was performed on PD-10 columns (Amersham Biosciences).

Electrophoretic analysis

Proteins were analysed by SDS/PAGE under reducing and non-reducing conditions using precast NuPAGE gels (Novex,

Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. Proteins were visualized by silver staining.

Amino acid analysis

In-gel digestion and extraction

The 75 kDa band from one lane in a Coomassie Blue-stained gel was excised and minced into small pieces. The gel pieces were washed with distilled water, dehydrated with acetonitrile, and dried under vacuum. The sample was rehydrated and digested with a digestion buffer containing 50 mM NH_4HCO_3 , 5 mM CaCl_2 and 12.5 $\mu\text{g/ml}$ sequencing-grade modified trypsin (Promega, Madison, WI, U.S.A.) overnight at 37 °C. The supernatant was collected, and the peptides were extracted from the gel pieces with 20 μl of 25 mM NH_4HCO_3 and several times with 20 μl of 50% (v/v) acetonitrile/5% (v/v) formic acid. Supernatants from each extraction were combined.

MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectrometry

The tryptic digest was analysed by MALDI-TOF MS (Kompact MALDI 4; Kratos). The mass analyser was scanned over an *m/z* range of 500 to 4000 atomic mass units, and the resulting spectra were used to search for matching proteins in the NCBI database using the Mascot search program (Matrix Science).

Nanoelectrospray MS (MS/MS)

After the initial peptide scanning, one peptide was subjected to MS/MS analysis (Micro Q-TOF, Manchester, U.K.), followed by searching with the fragmentation spectra in the NCBI database using the Mascot search program (Matrix Science).

Protein determination

Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as a standard according to the manufacturer's protocol.

Enzyme assay

The incubation mixture consisted of 0.1 ml of 100 mM sodium acetate, pH 7.0, 0.05 ml of 31 mM *N*-acetylglucosamine 6-sulphate containing 100 μg of sodium azide, and 0.05 ml of enzyme in 0.15 M NaCl at 37 °C. Since the formation of the product (*N*-acetylglucosamine) was linear with time for at least 24 h under standard conditions, the reaction mixture was incubated for 24 h and the reaction was stopped by adding 0.3 ml of water. The mixture was then added to 1 ml of Dowex AG 1-X 8 (200–400 mesh, chloride form; Sigma, Bellefonte, PA, U.S.A.). The void volume and three 0.5 ml fractions, eluted with 1.5 mM NaCl, were analysed for released *N*-acetylglucosamine.

The method of Reissig et al. [23] was used for the detection of *N*-acetylglucosamine released in the enzyme assay described below. Briefly, a 0.125 ml sample was heated in boiling water for 3 min after the addition of 0.025 ml of a potassium tetraborate reagent, which was 0.6 M with respect to borate (B_4O_7), adjusted to pH 9.5 with concentrated HCl. Next, the sample was cooled for 3 min in a room-temperature water bath followed by the addition of 0.75 ml of DMAB (*p*-dimethylaminobenzaldehyde) reagent [1 g of DMAB dissolved in 10 ml of acetic acid containing 12.5% (v/v) 10 M HCl and then diluted 1:9 (v/v) with acetic acid just prior to use]. The sample was then incubated in a 37 °C water bath for 20 min. It was again cooled for 3 min in a room-temperature

water bath, and the absorbance was measured at 585 nm with a Hitachi U-1100 spectrophotometer.

Analyses of pH optimum, K_m and V_{max}

The purified protein (0.277 μg) was added to tubes containing *N*-acetylglucosamine 6-sulphate at various pH values (4.0–10). The formation of product was assayed by the method of Reissig et al. [23], as described above. K_m and V_{max} values were obtained from Lineweaver–Burk plots with an *N*-acetylglucosamine 6-sulphate concentration range of 0.05–7.8 mM.

Substrate specificity

The substrate specificity was determined by incubation of the enzyme (0.50 μg) with the following substrates under standard conditions: *N*-acetylglucosamine 6-sulphate, glucose 6-sulphate, glucose 3-sulphate, galactose 4-sulphate, galactose 6-sulphate and *N*-acetylgalactosamine 6-sulphate (all purchased from Sigma Chemical Co.). The product formed was separated on a Dowex AG 1-X 8 column as described above and measured either by the method of Reissig et al. [23] or by the Park–Johnson assay [24].

HEK (human embryonic kidney) 293 cells, overexpressing 6-O-sulphotransferase, were metabolically ^{35}S -labelled for 24 h with 200 $\mu\text{Ci/ml}$ $\text{Na}_2[^{35}\text{S}]\text{SO}_4$ (Amersham Biosciences). ^{35}S -labelled proteoglycans were isolated as described [25]. To release the polysaccharide chains from the peptide core of the proteoglycan, the sample was treated with 0.5 M NaOH at 4°C for 20 h. After neutralization with 4 M HCl, the polysaccharide chains were isolated by gel chromatography. Heparan [^{35}S]sulphate was then purified [25] and treated with HNO_2 , pH 1.5 [26]. The ^{35}S -labelled deamination products were reduced with NaBH_4 and fractionated by gel chromatography on a column (1 cm $\times$ 180 cm) of Sephadex G-15 eluted with 0.2 M NH_4HCO_3 . The resultant [^{35}S]tetrasaccharides were pooled, lyophilized and fractionated further on a ProPac PA1 anion-exchange HPLC column (Dionex) using a gradient of 0–1 M NaCl, pH 3.0, at a flow rate of 1.0 ml/min. Di-O- ^{35}S sulphated tetrasaccharides were recovered, desalted, and dissolved in water. Non-reducing-end glucuronic acid and iduronic acid residues were eliminated by digestion with bovine liver β -D-glucuronidase (5 mg/ml; Sigma; this enzyme preparation also contained active α -L-iduronidase) in 50 mM sodium acetate, pH 5.0, containing 100 $\mu\text{g/ml}$ BSA in a total volume of 25 μl . After 24 h at 37°C, 87.5 μl of 100 mM sodium acetate, pH 7.0, 25 μl of 0.15 M NaCl, 1.5 μl of 0.2 M NaOH and 25 μl of purified 75 kDa protein (10 $\mu\text{g/ml}$ in 0.15 M NaCl) were added to a total volume of 200 μl and incubated overnight at 37°C. Control sample was incubated as above, except that the 75 kDa protein was omitted. Reactions were stopped by boiling the samples for 1 min, and the products were analysed on the ProPac PA1 anion-exchange column.

Alternatively, chemically [^{14}C]acetyl-labelled mono-O-sulphated tetrasaccharides were used as substrates for the 75 kDa protein, and products were analysed as above.

Subcellular fractionation

Blood cells were separated by density gradient centrifugation over isotonic 67 % (v/v) Percoll (Amersham Biosciences). The interphase, containing the mononuclear cells and lymphocytes, was removed. The pellet fraction, containing erythrocytes and granulocytes, was treated for 15 min with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.4) to lyse the erythrocytes, followed by hypotonic lysis of residual erythrocytes. The remaining granulocytes were washed twice in PBS (without Ca^{2+}). To further separate neutrophils from eosino-

phils, the isolated granulocytes were incubated for 1 h at 4°C with anti-CD16 monoclonal antibody-coated magnetic microbeads [1×10^7 granulocytes in 30 μl of PBS containing 2 % (v/v) newborn calf serum to 15 μl of microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany]. The cells were subsequently allowed to pass through a steel matrix column in a magnetic field. After removing the magnetic field, neutrophils were eluted with PBS, washed and suspended in 6 % (w/v) sucrose solution for ultrasonication. The purity of the neutrophils was > 98 %. Isolated neutrophils were resuspended in 3 ml of 6 % (w/v) sucrose solution at approx. 1×10^8 cells/ml. Ultrasonication was used to disrupt the neutrophils. The ultrasonicate was mixed with 0.2 ml of 32 % (w/v) sucrose. The nuclei and intact cells were pelleted by centrifugation at 450 g for 20 min. The postnuclear supernatant was loaded on to a three-layer discontinuous sucrose density gradient, consisting of a 60 % cushion overlaid with 50 % and 40 % (w/v) sucrose solutions [all solutions were prepared in 10 mM Hepes, pH 7.4, plus 0.5 mM PMSF (Sigma)]. The sucrose gradient was spun in a nSW 28.1 rotor (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.) at 28 000 rev./min for 2 h. The gradient was collected in 32 fractions of 0.5 ml from the top of the tube.

Separation of organelle matrix proteins

To separate organelle matrix proteins from membrane proteins, neutrophil organelles were submitted to five cycles of freeze–thawing. The supernatant containing the organelle matrix proteins was aspirated after centrifugation at 100 000 g for 90 min. The pellet was resuspended in solubilization buffer [0.5 % (v/v) Triton X-100, 10 mM Hepes, pH 7.4] containing 0.5 mM PMSF and was kept at room temperature for 1 h. The mixture was centrifuged at 100 000 g for 30 min, and the supernatant containing the membrane proteins was aspirated. The resulting organelle matrix proteins and membrane proteins were tested for enzymatic activity.

Release study

Isolated neutrophils were resuspended in Hanks balanced salt solution at approx. 1×10^8 cells/ml and stimulated with PMA (5 $\mu\text{g/ml}$) for 20 min at 37°C. The activated neutrophils were pelleted by centrifugation and the released material was aspirated. The cell pellet was resuspended in solubilization buffer (as above) containing 0.5 mM PMSF and was kept for 1 h at room temperature. After centrifugation (150 000 g for 120 min), the supernatant was collected. Quantification of enzyme activity in the released material was expressed as percentage of the total enzyme activity (i.e. that in released material + pellet).

Marker protein assays

Radio-immunoassays of myeloperoxidase [27] and HNL (human neutrophil lipocalin) [28] were performed as described. Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate as substrate, as described in [29].

RESULTS AND DISCUSSION

Purification of a 75 kDa protein

A 75 kDa protein was purified from acid extracts of granules from normal human granulocytes by means of a three-column procedure consisting of Sephadex G-75 chromatography, Mono-S cation exchange chromatography and Mono-P chromatofocusing (Table 1).

Table 1 Purification of *N*-acetylglucosamine-6-sulphatase from acid extracts of granules from normal human granulocytes

Purification step	Total protein (mg)	Total activity (nM/min)	Specific activity (nM/min per mg)	Recovery (%)	Purification (fold)
Acid extracts	99.4	169.2	1.7	100	1
Superdex G-75	6.8	121.8	17.9	72	11
Mono-S (pH 4.0)	0.152	88.4	581.6	52	342
Mono-S (pH 5.0)	0.104	83.0	798.1	49	469
Mono-P	0.076	66.4	922.2	39	542

Figure 1 Separation of granule proteins by gel filtration and ion-exchange chromatography

(A) Acid extracts of granules obtained from human granulocytes were loaded on a Sephadex G-75 column (2.5 cm $\times$ 90 cm) and eluted with 0.2 M sodium acetate, pH 4.5, as described in the Experimental section, giving peaks A–E. As indicated, the fractions in the peaks (A–E) were pooled (pools 1–5). The majority of the 75 kDa protein was contained in pool 2(a) of peak B (fractions 68–76), as indicated by the horizontal bar, as judged by SDS/PAGE after further separation of proteins in each pool on a Mono-S column (results not shown). (B) Ion-exchange chromatography was performed as described in the Experimental section. Fractions 68–76 from the gel filtration column were applied to the Mono-S column and eluted by a linear gradient from 0 to 1.0 M NaCl in 0.1 M sodium acetate, pH 4.0. The 75 kDa protein was eluted in fractions 11–17 of peak 2, as indicated by the horizontal bar.

Granule acid extracts were first separated by gel filtration chromatography. The 75 kDa protein was eluted on a Sephadex G-75 column equilibrated with 0.2 M sodium acetate, pH 4.5, in pool 2(a) (Figure 1A). The proteins in fractions 68–76 were applied to a Mono-S cation-exchange column equilibrated with 0.1 M sodium acetate, pH 4.0, and eluted with a linear gradient from 0 to 1.0 M NaCl in 0.1 M sodium acetate, pH 4.0. The 75 kDa protein was eluted at a NaCl concentration of approx. 0.25 M in peak 2 (fractions 11–17) (Figure 1B). The fractions containing the 75 kDa protein were loaded on to the same Mono-S column equilibrated with 0.02 M sodium acetate, pH 5.0, and the proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 0.02 M sodium acetate, pH 5.0, resulting in two major peaks. As shown in Figure 2(A), the 75 kDa protein was contained in peak B, while

Figure 2 Purification of the 75 kDa protein by ion-exchange chromatography and chromatofocusing

(A) Fractions 11–17 of peak 2 from the Mono-S column were applied to the same column equilibrated with 0.02 M sodium acetate, pH 5.0, and eluted by a linear gradient from 0 to 0.5 M NaCl in 0.02 M sodium acetate, pH 5.0. The 75 kDa protein was eluted in peak B, as indicated (horizontal bar). (B) The fractions containing the 75 kDa protein from the second Mono-S column were applied to a Mono-P prepacked column equilibrated with 0.025 M diethanolamine, pH 9.5, and eluted with Polybuffer 74, pH 4.0. The fractions, as indicated below the chromatogram, were collected as pure protein.

most contaminants passed through the column. The proteins in peak B were separated further on a Mono-P chromatofocusing column as shown in Figure 2(B). The 75 kDa protein was eluted as one major peak consisting of three narrowly spaced peaks, at a pH around 5.4, when eluted with Polybuffer 74, pH 4.0.

Samples of approx. 0.1–10 μ g of protein from steps one to four of the purification procedure were applied to SDS/PAGE, and proteins were visualized by silver staining. The purified protein from step four of the purification gave only one band at a molecular mass of 75 kDa under non-reducing (Figure 3A, lane 6) and reducing (Figure 3B, lane 2) conditions, indicating a single polypeptide of the 75 kDa protein.

Amino acid analyses

In order to identify it, the purified 75 kDa protein from SDS/PAGE was digested by trypsin, followed by MALDI-TOF and MS/MS analyses. The resulting spectrum was used to search for matching proteins in the NCBI database, using the Mascot search program. The search yielded a top score of 119 for *N*-acetylglucosamine-6-sulphatase (protein scores greater than 67 are significant; $P < 0.05$). The amino acid residues identified by MALDI were 46–68, 126–137, 150–177, 220–246, 247–255, 293–305, 342–349, 389–401, 410–421 and 494–502. Furthermore, the selected tryptic peptide (m/z 1398.78) sequenced by MS/MS revealed an amino acid sequence of IQEPNTFPAILR, corresponding to residues 126–137 of *N*-acetylglucosamine-6-sulphatase.

Enzyme characteristics

The purified 75 kDa protein showed enzyme activity with an optimum at pH 7.0 when *N*-acetylglucosamine 6-sulphate was used

Figure 3 SDS/PAGE of material from each step of the chromatographic purification procedure

After each purification step, approx. 0.1–10 μ g of protein was applied to SDS/PAGE under non-reducing (A) and reducing (B) conditions, and analysed by silver staining as described in the Experimental section. Marker proteins and their corresponding molecular masses are indicated in lanes 1. (A) Lane 2, material from acid extracts of granules; lane 3, material from Sephadex G-75 purification step; lane 4, material from first Mono-S purification step at pH 4.0; lane 5, material from second Mono-S purification step at pH 5.0; lane 6, material from Mono-P purification step. (B) Lane 2, material from Mono-P purification step.

Table 2 Substrate specificity of *N*-acetylglucosamine-6-sulphatase

Each substrate was incubated with the enzyme under standard conditions, as described in the Experimental section. The release of *N*-acetylglucosamine from the substrate was determined by the method of Reissig et al. [23], and the release of glucose, galactose and *N*-acetylgalactosamine from their respective substrates was determined using the Park–Johnson assay [24].

Substrate	Sugar released (%)
<i>N</i> -Acetylglucosamine 6-sulphate	24.0
Glucose 6-sulphate	13.1
Glucose 3-sulphate	0
Galactose 4-sulphate	0
Galactose 6-sulphate	0
<i>N</i> -Acetylgalactosamine 4-sulphate	0

as substrate at 37 °C. The enzyme showed classical Michaelis–Menten kinetics, and from Lineweaver–Burk plots a K_m of 13.0 mM and a V_{max} of $\sim 1.8 \mu$ M/min per mg were calculated. The specificity of the enzyme towards a variety of commercially available sulphated sugars is shown in Table 2. The hydrolysis of the sulphated sugars was measured with pure enzyme, which was very active against *N*-acetylglucosamine 6-sulphate and glucose 6-sulphate.

To address further the specificity of the 75 kDa protein, labelled heparan sulphate-derived oligosaccharides were used as substrates. HEK 293 cells were metabolically labelled with [35 S]-sulphate, and 35 S-labelled heparan sulphate tetrasaccharides were isolated for enzymatic analysis. As a result of the method used in the preparation, the tetrasaccharides have the backbone structure GlcA[β 1-4]IdoA[α 1-4]GlcNAc[α 1-4]GlcA[β 1-4]aMan_R

Figure 4 Release of 6-O-[35 S]sulphate groups from heparan sulphate oligosaccharides by the 75 kDa protein

(A) Metabolically 35 S-labelled mono- and di-O- 35 S-sulphated tetrasaccharides were separated by ProPac chromatography and fractions corresponding to di-O- 35 S-sulphated tetrasaccharides were pooled, as indicated by the horizontal bar, and desalted. (B) The purified di-O- 35 S-sulphated tetrasaccharides were then digested with α -iduronidase/ β -glucuronidase, followed by incubation with the 75 kDa enzyme, and the products were analysed on a ProPac PA1 column. The minor peak at 34 ml most probably corresponds to di-O-sulphated tetrasaccharide with a 2-O-sulphated iduronic acid at the non-reducing end that is resistant to digestion with α -iduronidase. The column was eluted with a linear gradient indicated by the broken lines. Effluent fractions of 0.5 ml were analysed for radioactivity. Elution positions of standards are indicated: 1, inorganic sulphate ($^{35}\text{SO}_4^{2-}$) and mono-6-O- 35 S-sulphated trisaccharide; 2, di-6-O- 35 S-sulphated trisaccharide.

(aMan_R, i.e. 2,5-anhydro-D-mannitol, is formed by reduction of terminal 2,5-anhydromannose residues with NaBH₄). 6-O-[35 S]-sulphate groups are located at GlcNAc and aMan_R residues. Anion-exchange HPLC (ProPac column) of the purified tetrasaccharide revealed two major peaks, corresponding to labelled mono-O-[35 S]sulphated and di-O-[35 S]sulphated species (Figure 4A). Digestion of the purified di-O-[35 S]sulphated tetrasaccharide with a combination of β -glucuronidase and α -iduronidase resulted in the formation of di-6-O-[35 S]sulphated trisaccharides (caused by removal of the non-reducing-end hexuronic acid residue from the tetrasaccharides; results not shown). Subsequent digestion with the purified sulphatase resulted in a major release of label from the tetrasaccharide fractions, with concomitant formation of inorganic [35 S]sulphate and mono-O-[35 S]sulphated trisaccharides (Figure 4B). No release of inorganic [35 S]sulphate was observed in control incubations without added sulphatase (results not shown). To corroborate the above findings, similar experiments were performed using mono-O-sulphated [14 C]acetyl-labelled tetrasaccharides. Approx. 20 % of the mono-O-sulphated [14 C]acetyl-labelled material was converted into non-sulphated trisaccharides. No formation of non-sulphated products occurred in control samples without the addition of the sulphatase (results not shown). Taken together, these results show that the sulphatase apparently can act on heparan sulphate-derived oligosaccharides in addition to monosaccharide substrates.

Figure 5 Subcellular distribution of *N*-acetylglucosamine-6-sulphatase in human neutrophils

(A) Resting neutrophils were disrupted and fractionated on a sucrose density gradient, as described in the Experimental section, into three fractions γ , β and α . (B) Each fraction was analysed for *N*-acetylglucosamine-6-sulphatase activity. The amount of *N*-acetylglucosamine-6-sulphatase activity in each fraction was expressed as a percentage of the total activity in all three fractions γ , β , and α . Data are shown from one representative experiment. AP, alkaline phosphatase; MPO, myeloperoxidase.

Subcellular localization of *N*-acetylglucosamine-6-sulphatase in neutrophils

To investigate the subcellular localization of *N*-acetylglucosamine-6-sulphatase in human neutrophils, the neutrophil postnuclear supernatant was fractionated on a sucrose density gradient and the positions of plasma membranes/secretory vesicles, secondary granules and primary granules were identified by the marker proteins [30–32] alkaline phosphatase (γ -band; fractions 6–10), HNL (β -band; fractions 15–19) and myeloperoxidase (α -band; fractions 22–26) respectively on the gradients (Figure 5A). The subcellular distribution of *N*-acetylglucosamine-6-sulphatase among these fractions was subsequently determined by measuring its activity (Figure 5B). The distribution profile of *N*-acetylglucosamine-6-sulphatase activity in resting neutrophils was estimated to be approx. 11% in the fractions of plasma membranes/secretory vesicles, 26% in the fractions of secondary granules, and 63% in the fractions of primary granules.

N-Acetylglucosamine-6-sulphatase in the organelle matrix and its release

To establish the presence of *N*-acetylglucosamine-6-sulphatase in the neutrophil organelle matrix, matrix proteins were separated from membrane-bound proteins by freeze–thawing of isolated neutrophil organelles and centrifugation. More than 68% of *N*-acetylglucosamine-6-sulphatase activity appeared to be in the preparation of organelle matrix proteins after only five freeze–thawing cycles. To investigate the mobilization of *N*-acetylglucosamine-6-sulphatase from human neutrophils upon stimulation, isolated neutrophils were stimulated with PMA. The released materials and the cell pellets were tested for *N*-acetylglucosamine-6-sulphatase activity. As shown in Table 3, *N*-acetylglucosa-

Table 3 Release of myeloperoxidase, HNL and *N*-acetylglucosamine-6-sulphatase upon activation of neutrophils by PMA

Cells were resuspended in Hanks balanced salt solution at approx. 1×10^8 cells/ml and stimulated with PMA for 20 min at 37°C. Control cells were kept for 20 min at 37°C. Data are means $\pm$ S.E.M. from three independent experiments.

Treatment	Release (%)		
	Myeloperoxidase	HNL	<i>N</i> -Acetylglucosamine-6-sulphatase
Control	0.9 ± 0.2	3.1 ± 0.3	1.0 ± 0.1
PMA ($5 \mu\text{g/ml}$)	5.1 ± 1.5	56.5 ± 0.5	7.7 ± 0.3

mine-6-sulphatase activity could be detected in the released materials, confirming that the enzyme is a matrix protein in the mobilizable organelles of human neutrophils. In contrast with the extensive release of the secondary granule protein HNL, the *N*-acetylglucosamine-6-sulphatase activity released represented < 8% of the total cellular content, which is compatible with its major localization in the primary granules, and similar in extent to the release of myeloperoxidase, another primary granule constituent.

Conclusions

We have purified a 75 kDa protein, identified as *N*-acetylglucosamine-6-sulphatase, from granule extracts of normal human granulocytes by means of a simple three-column procedure. The protein consists of one chain with a pI of approx. 5.4. The protein is a matrix protein that was found mainly in fractions containing primary granules of human neutrophils. The protein was identified as an *N*-acetylglucosamine-6-sulphatase by amino acid sequence analysis and enzymatic activity towards a specific substrate, *N*-acetylglucosamine 6-sulphate. However, it is obvious that *N*-acetylglucosamine-6-sulphatases purified from different sources differ in molecular mass, isoelectric point and enzyme characteristics. The urinary enzyme was reported to have a molecular mass of 97 kDa with a pI of 5.4–8.3. The major form of the liver-derived enzyme has a molecular mass of 78 kDa with a pI of > 9.5. The urinary and liver-derived enzymes showed pH optima of 5.5 and 5 respectively, exhibiting typical features of a lysosomal hydrolase (acid pH optimum) [8,9], whereas the neutrophil-derived enzyme exhibits a neutral pH optimum. The function of the enzyme in human neutrophils is not known. Its localization in mobilizable organelles of human neutrophils and the neutral pH optimum suggest an extracellular O-desulphation role.

Heparan sulphate is ubiquitous in the extracellular matrix and on cell surfaces [33]. Due to its negative charge, heparan sulphate interacts with a variety of proteins, such as cytokines, extracellular matrix proteins, enzymes and enzyme inhibitors [34]. Heparan sulphate on the host cell surface has been implicated in the adherence of numerous microbial agents [35]. Various studies suggest that the sulphation states of glucosamine residues in heparan sulphate moieties of proteoglycans influence their activities in the interaction with specified proteins and microbial agents [15–18]. Therefore it is possible that neutrophils play a role in the modulation of cell surface molecules and extracellular matrices by O-desulphation.

In summary, we have described the purification and characterization of a 75 kDa protein, *N*-acetylglucosamine-6-sulphatase, from normal human granulocytes. The function of the enzyme in human neutrophils remains to be determined. However, the localization of the enzyme in the matrix of mobilizable organelles of human neutrophils suggests an extracellular role involving

the desulphation of proteoglycans. The availability of the neutrophil-derived enzyme will enable us to define further the functions of *N*-acetylglucosamine 6-sulphate in relation to sulphated proteoglycans *in vivo* and *in vitro*.

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