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Purification and characterization of Kurloff cell sialoglycoproteins with acid phosphatase activity

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The major α2–6 sialoglycoproteins in detergent-extracts of Kurloff cells were purified by anion-exchange and *Sambucus nigra* agglutinin-affinity chromatographies. The similar ultrastructural localisations of (1) *S. nigra* agglutinin-gold conjugates and (2) acid phosphatase activities on the Kurloff body and particularly on its myelin figures indicated that the major α2–6 sialoglycoproteins of the Kurloff cell had acid phosphatase activity. Two-dimensional electrophoresis showed that these tartrate-sensitive phosphatases corresponded to 2 acidic (pI 3.4–3.7) polypeptides of 36 and 34 kDa. Hydrolysis with peptide-*N*-glycosidases F gave a 33 kDa apoprotein rich in alanine, glutamic acid, tyrosine and lysin. A lectin-affinity study demonstrated that they contained hybrid type bisected and fucosylated *N*-linked oligosaccharides. Cytotoxic properties were previously attributed to Kurloff cells and other studies suggested that not only acid phosphatases but also α2–6-linked sialic acid residues themselves may participate in natural killer activity.

Keywords: Kurloff cell, acid phosphatases, sialoglycoproteins, lectin-blotting, lectin chromatography, natural killer

Introduction

Kurloff cells (for review see [1]) were described as cytotoxic lymphocytes with natural killer (NK) activity [2] or NK-like activity [3]. Kurloff cells contain a very large (10 μm in diameter) granule, the Kurloff body (KB). The Toluidine Blue metachromasia of the KB has been linked to the presence of protease-resistant proteochondroitin-4-sulphate [4]. Cytochemical [5–7] and zymographic [8, 9] studies have shown that the KB are rich in acid hydrolases. The lectin from *Sambucus nigra* (SNA), which recognizes the sequence Neu5Ac(α2–6)Gal/GalNAc [10], was used to localise α2–6-linked sialic acids in KC [11] and to demonstrate their presence in the major glycoproteins of KC [12]. Most of the 30–35 kDa α2–6 sialoglycoconjugates appear to be the most acidic isoforms of acid phosphatases. Other sialoglycoconjugates with both α2–8 polysialic acid and α2–6-linked sialic acid isomers were detected in the highly anionic, non-sulphated fraction in the molecular weight range 100 kDa [13]. Similar polysialic chains have been described in N-CAM and CD56 molecules [14].

Numerous studies have indicated that carbohydrates or glycoconjugates are involved in various stages of the cytolytic cascade of NK cells. There is evidence that α2–6-linked sialic acid residues may help mediate NK cytolysis [15]. There also appear to be increases in both the sialylation of KC glycoproteins and of KC during the development of acute lymphoblastic leukemia in S2 guinea-pigs (Taouji, unpublished data). Hence, a detailed knowledge of the structure of the carbohydrate chains of these α2–6 sialoglycoconjugates is required. This report describes the separation of the weakly anionic fraction containing the low molecular weight α2–6-sialoAcPases, from the highly anionic, highly sialylated fraction by anion-exchange chromatography. The former sialoglycoconjugates were then purified using the binding of *S. nigra* agglutinin to Neu5Ac(α2–6)Gal|GalNAc units. Finally, structure of KC sialophosphatases was examined using lectin-affinity and glycosidases.

Materials and methods

ACID PHOSPHATASE ASSAY

AcPase activities were determined using p-nitrophenyl-phosphate in a microtitre plate. The assay buffer was 2.5 M sodium citrate, pH 4.8. The reaction mixture was

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incubated for 15 min at 37 °C and the reaction stopped by adding 1 M NaOH.

PROTEIN ASSAY

Proteins were determined with the Bio-Rad detergent compatible protein assay kit (DC protein assay) using BSA as standard, in accordance with the manufacturer's instructions.

SIALIC ACID ASSAY

Sialic acid content was determined according to Simpson *et al.* [16].

EXTRACTION OF DETERGENT-SOLUBLE KC GLYCOCONJUGATES

KC were isolated according to Landemore *et al.* [17] and suspended in ice-cold buffer (0.05 M Tris-HCl (pH 6.8) containing: caproïc acid (100 mM); benzamidine (5 mM); EDTA (10 mM) and 1% Triton X-100) and homogenized using a Dounce homogenizer. Some cells were homogenized immediately after isolation, others were incubated with L-[^{14}C] methionine before homogenization. The resulting homogenates were centrifuged at 100 000 g for 60 min at 4 °C and the supernatant (detergent-soluble extract) was saved.

ONE- AND TWO-DIMENSIONAL ELECTROPHORESIS

The detergent-soluble extracts were subjected to one-dimensional SDS-gradient 4–15% PAGE according to Laemmli [18]. Samples for two-dimensional electrophoresis were separated by isoelectric focusing in tube gels at 500 V for 16 h according to O'Farrell [19]. The tube gels (3 mm internal diameter) contained: 9 M urea, 4% acrylamide (acrylamide: bisacrylamide ratio 25:1), 2% Nonidet P-40, 7% ampholines (pH 3–10: pH 5–7, ratio 1:6). These gels were then placed in 50 mM Tris, pH 6.7, 4% glycerol, 2% SDS, 2% 2-mercaptoethanol, with Bromophenol Blue as a marker for 15 min at room temperature, placed on top of SDS-PAGE slab gels and electrophoresed in the second dimension. The electrophoresis conditions used resolved proteins with isoelectric points at 3.5–6.5, based on isoelectric point standards (Pharmacia) and on the pH of the tube gels, measured directly by homogenizing 0.5 cm sections of the gels in water and determining their pH with a pH meter.

PREPARATION OF HIGHLY ANIONIC KC SIALOGLYCOCONJUGATES BY ANION-EXCHANGE H.P.L.C:

Detergent-soluble extracts were applied to a TSK-DEAE column equilibrated with 50 mM Tris-HCl (pH 6.8) containing protease inhibitors (2 mM PMSF, 10 mM EDTA, 5 mM caproic acid). The column was washed thoroughly with equilibrium buffer, and proteins were eluted with a linear NaCl gradient (0–1 M) in equilibrium buffer. The flow rate throughout was 2 ml min^{-1}. Proteins were detected by absorption at 280 nm. Each 1 ml fraction was also monitored for radioactivity. The unbound fractions containing acid phosphatase activity were dialyzed against cold water, lyophilized and analysed by SDS-PAGE.

PRECIPITATION, WITH *S. NIGRA* AGGLUTININ, OF α2–6 SIALOGLYCOCONJUGATES FROM THE WEAKLY ANIONIC FRACTION AND CONTROL OF THE RESIDUAL AcPase ACTIVITY OF THE SUPERNATANTS

Aliquots of the washout (200–500 µg protein) from anion-exchange chromatography were incubated and precipitated with increasing amounts of SNA (0–1.5 mg protein ml^{-1}), for 2 h at 4 °C in 5 M NaCl, 0.2 M Tris buffer, pH 7.4. The mixtures with complexed glycoproteins were centrifuged (5000 g, 15 min). The pellets were washed twice with the same buffer plus SNA and their glycoproteins were analysed by SDS-PAGE electrophoresis with or without reducing agents. The AcPase activity of the supernatants was assayed with p-nitrophenyl-phosphate. Control experiments with sialyllactose (0.1 M) added to the samples, were also carried out.

LECTIN-AFFINITY CHROMATOGRAPHY OF α2–6 SIALOGLYCOCONJUGATES (SGP35)

The α2–6 sialoglycoconjugates (SGP35) with AcPase activity were isolated from the DEAE-washout by affinity chromatography on immobilized SNA.

Coupling the lectin to the support

A column of ImmunoPure^{R} immobilized streptavidin on agarose (Pierce) (2 mg streptavidin/ml swollen gel) was equilibrated with 0.5 M NaCl in 20 mM PBS, pH 7.5. Biotinylated SNA (2 mg ml^{-1}) was run onto the column and incubated for 1 h. Protein determinations of buffer washes indicated that >80% of the biotinylated lectin had reacted with the immobilized streptavidin.

Chromatography

Pooled washout from anion-exchange chromatography (4×10^5 cpm) was dissolved in 50 µl 20 mM PBS, pH 7.5 containing 0.02% NaN_3, 0.5 M NaCl and placed on a 1 ml column containing SNA-streptavidin-agarose, equilibrated with the same buffer at room temperature. AcPase were eluted with a 0–100 mM lactose gradient and fractions (0.4 ml) were collected at a flow rate of 18 ml h^{-1}. Elution was monitored by measuring radioactivity or AcPase activity.

To check that the immobilized lectin was not saturated during the first run, the non-reactive compounds went through a second cycle of adsorption and elution on the same column. The efficiency of the chromatography was monitored by lectin staining, according to the manufacturer's instructions (Boehringer-Mannheim), following

spotting aliquots of the lectin reactive and non-reactive fractions on nitrocellulose strips.

ConA lectin affinity chromatography of SGP35 was performed as described by Oulhaj *et al.* [20]. Briefly, pooled bound fractions from the SNA-column (2×10^5 cpm) were desalted and concentrated on a Millipore membrane (cutoff 5000) and dissolved in 50 μl ConA buffer (50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 M NaCl and 0.02% NaN_3. The unbound fractions were layered onto a Sepharose-ConA column (2×0.5 cm) and eluted with ConA buffer alone, followed by buffers containing 10 mM, and 200 mM methyl-α-D-mannopyrannose.

OLIGOSACCHARIDES IN THE SGP35

Dot-blots and Western-blots [12] were used to test the reactivities with digoxigenin- or biotin-labelled lectins using the Lectin Differentiation kit (Boehringer) and Lectin-Link kit (Genzyme).

DEGLYCOSYLATION OF SGP35

Aliquots of SGP35 (20–25 μl, ~2 μg protein) were denatured by heating in a boiling-water bath for 10 min and cooled to room temperature. Denatured samples were incubated with 15 μl deglycosylation buffer (15 mM phosphate buffer, pH 7.0, 10 mM EDTA, 2 mM PMSF, and 1% (v/v) 2-mercaptoethanol) and 0.25 unit peptide-N-glycosidase F (Boehringer-Mannheim) at 37 °C overnight. A second 0.25 unit glycosidase was then added, and incubation was continued for a further 24 h.

AMINO ACID ANALYSIS OF SGP35

Amino acid analysis of SGP35, was performed according to Desgres *et al.* [21]. Briefly, purified SGP35 from Sephadex G25 chromatography was hydrolysed, esterified, acetylated and analysed on a Chromosorb W, AW DMCS G.L.C. column. Amino acids were identified by their elution times and quantified by comparison with standards.

Results

MOLECULAR MASS DISTRIBUTION OF α2-6 SIALOGLYCOPROTEINS AFTER ONE- AND TWO-DIMENSIONAL ELECTROPHORESIS OF KC EXTRACTS

The molecular mass of KC α2–6 sialoglycoproteins, was determined by SDS-PAGE, Western blotting and SNA staining. The electrophoretic pattern of KC α2–6-linked sialoglycoconjugates showed a broad SNA-reactive band at 30–36 kDa and weaker bands at 80–110 kDa (Fig. 1; Fig. 2B, lane B). These two groups of SNA-reactive glycoproteins were designated SGP35 and SGP80. Two-dimensional electrophoresis and silver staining (Fig. 1A), revealed prominent protein spots at 30–36 kDa in the pI zone 3.4, and 80–110 kDa protein spots that were more weakly stained.

The pI 3.4 SGP35 were resolved into two spots at 34 and 36 kDa (Fig. 1B). The size microheterogeneity of the SGP80 was confirmed by SNA-staining and their charge homogeneity was established by their location pI 6.5 alone (80–110 kDa).

PURIFICATION AND CHARACTERIZATION OF SGP35

Anion-exchange chromatography

Almost all the ^{14}C methionine radiolabelled protein content in KC extract was recovered from a TSK-DEAE column in the unbound-fraction (peak I in Fig. 2A). This unbound material also had a high absorbance at 280 nm. The material eluted by 0.3–0.4 M NaCl absorbed strongly at 280 nm and at 220 nm (data not shown), but was weakly labelled with methionine (4% of the ^{14}C methionine in this bound fraction). Strongly bound material eluted at 0.5 M NaCl was detected only by a small absorbance peak.

Electrophoretic analysis revealed that the SGP35 were in the pooled unbound material and SGP80 in 0.3–0.4 M NaCl fraction (Fig. 2B, lanes D and E). The sialic acid concentration of the SGP35 fraction was 0.08 g sialic acid per g protein and the SGP80 contained 0.5 g sialic acid per g protein. However the SGP35 represented 65% of the deposited sialic acids. Despite their low pI, SGP35 were not strongly bound to the anion-exchange column.

Precipitation with SNA

The lectin-glycoprotein precipitate obtained by mixing and incubating 1 mg SNA with the DEAE column flow through, migrated as a broad band at 35 kDa in SDS-PAGE (reducing) (Fig. 3A, lane b), but has two bands: a minor one at 140 kDa zone and a major band at 33 kDa under non reducing conditions (Fig. 3A, lane d). SGP35, thus, appeared to have a higher electrophoretic mobility under non-reducing conditions than under the reducing ones. The minor 140 kDa band was the *S. nigra* lectin itself. This lectin is a tetramer of 34 and 37 kDa subunits held together by disulphide bridges [22]. No precipitate was obtained in the presence of 0.1 M sialyllactose.

The supernatants obtained after precipitation of these KC extracts with SNA (0–1.5 mg ml^{-1}) lost the AcPase activity of the starting extract (IC50 = 0.15 mg ml^{-1}) and reached a plateau at 5% residual activity (Fig. 3B). The slightly higher SNA concentration of 1 mg ml^{-1}, gave less inhibition (residual activity: 30%). Very high SNA concentrations (up to 1 mg ml^{-1}) produced the minimal residual activity (5%, for maximal inhibition).

Lectin affinity chromatography of SGP35

SGP35 was purified by affinity chromatography on immobilized SNA. SNA-bound material was eluted with

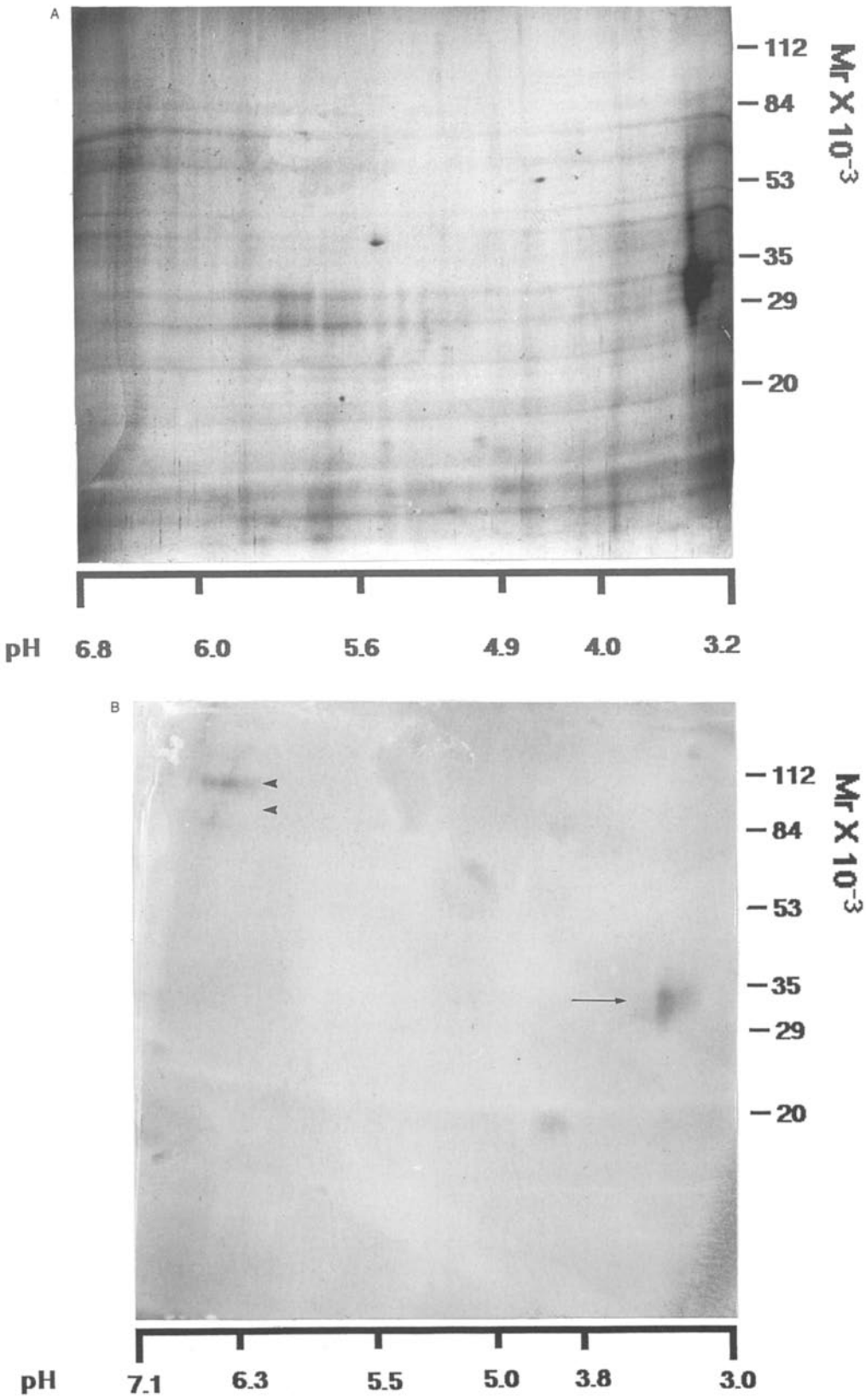

Figure 1. Detection of α2–6 sialoglycoproteins after two-dimensional electrophoresis of detergent-soluble glycoproteins from Kurloff cells. (A) Control silver staining for proteins. (B) Staining of α2–6 sialoglycoproteins with SNA-digoxigenin and alkaline phosphatase-antidigoxigenin conjugates after Western blotting onto Immobilon^R. SGP35 is indicated by an arrow and, SGP80 by an arrow-head.

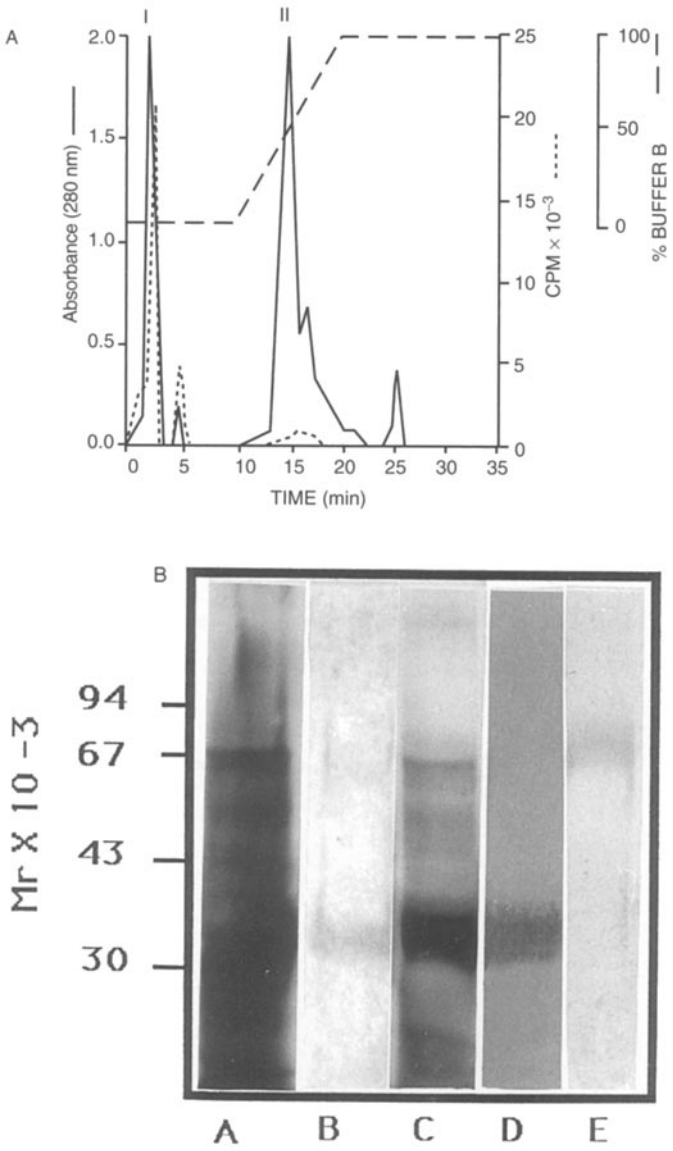

Figure 2. Separation of weakly anionic and strongly anionic α2–6 sialoglycoproteins by TSK-DEAE HPLC. (A) Anion-exchange chromatography of a detergent-soluble extract KC labelled with ^{14}C methionine. Running buffer B consisted of 1 M NaCl in equilibrium buffer. Aliquots (100 μl) of the effluent were assayed for radioactivity and protein. (B) SDS-PAGE and Western blotting of the SNA-reactive material (peak **I**) and bound highly anionic material (peak **II**). α2–6 sialoglycoproteins of the starting cytosolic extract (lane B), of peak **I** (lane D) and of peak **II** (lane E); total protein in the cytosolic extract (lane A) and in peak **I** (lane C) after silver staining.

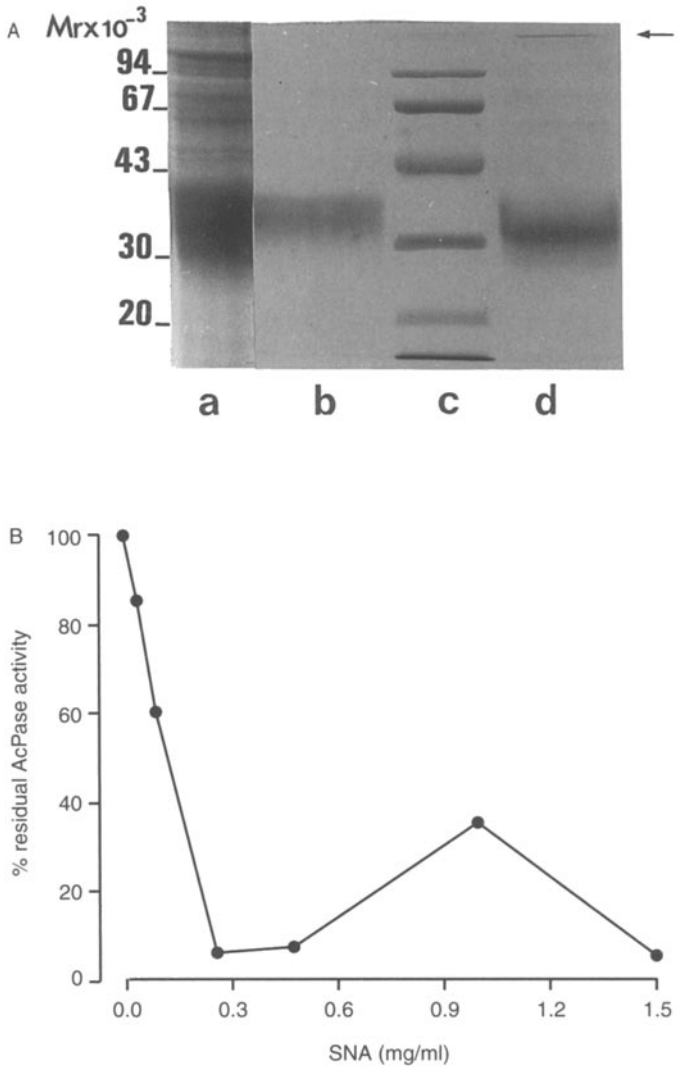

Figure 3. Precipitation of peak **I** by *S. nigra* agglutinin: electrophoretic analysis of the precipitate (A) and assay of residual AcPase activity (B). (A) SDS-PAGE of the precipitate under reducing (lane b) and non-reducing (lane d) conditions. The Coomassie Blue staining of the detergent-soluble extract is also shown (lane a). SNA is shown by an arrow-head (lane d). Mr values were estimated from molecular weight standards (lane c). (B) Residual hydrolysis of p-nitrophenyl phosphate by the supernatant obtained by precipitation of peak **I** with *S. nigra* agglutinin.

25 mM and 40 mM lactose as two major radioactive peaks (Fig. 4A). PAGE-SDS gave two sharp Coomassie Blue-stained bands at 34 kDa and 36 kDa (Fig. 5A, lane D), similar to that obtained by two-dimensional electrophoresis and SNA-blotting of the crude extract, or after precipitation with SNA.

Ninety per cent of the AcPase activity recovered was in the SNA-bound fractions (Table 1), which represented 3% of the deposited proteins. The enzyme was purified 61-fold with a recovery of about 60%. Most (75%) of the AcPase activity in KC was in the SNA-bound fractions, and these were tartrate-sensitive AcPases. Tartrate-resistant acid phosphatase (TRAP) activity, 30% of the total AcPase activity in KC, was detected only in the SNA-unbound fraction, with traces of tartrate-sensitive activity.

The structural features of *N*-linked oligosaccharides of

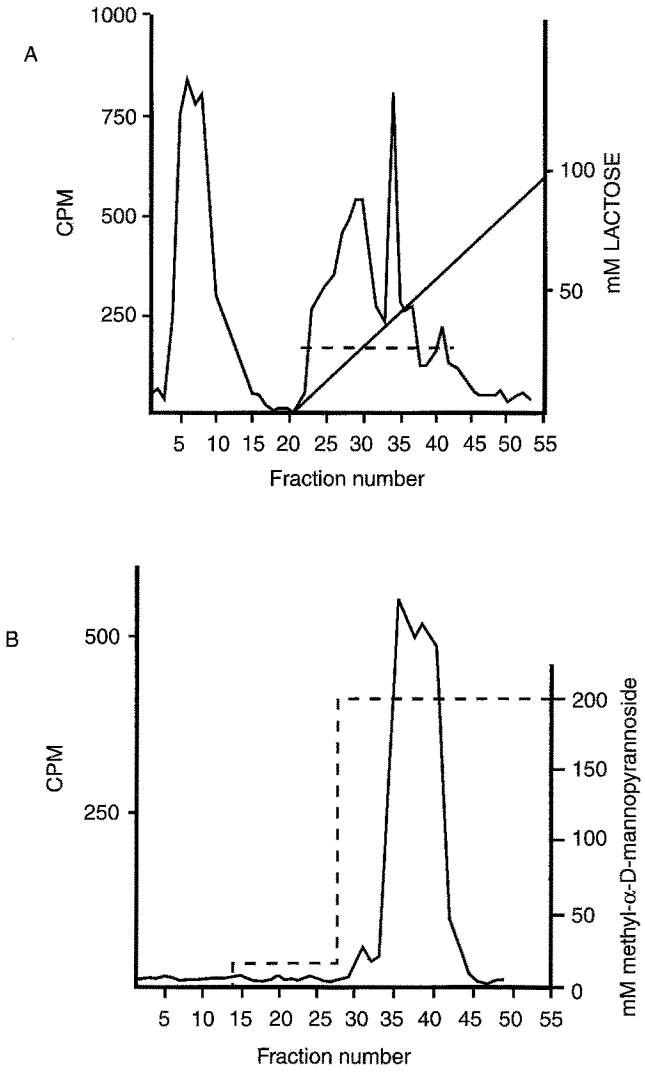

Figure 4. Purification of SGP35. Affinity chromatography on SNA-Sepharose (A) and ConA-Sepharose (B) columns. (A) ^{14}C methionine-labelled peak **I** was layered onto a 2 ml column of SNA-Sepharose equilibrated with 20 mM PBS, pH 7.5 containing 0.02% NaN_3, 0.5 M NaCl. The unbound material was drained with equilibrium buffer alone and the bound material was eluted with a linear 0–100 mM lactose gradient. (B) The SNA-bound material (fractions 21–42, indicated by a dashed line in Fig. 4A) were placed on a ConA-Sepharose column (1 ml) equilibrated with 50 mM Tris-HCl buffer/1 mM $CaCl_2$, 1 mM $MgCl_2$. No radioactive material was drained with equilibrium buffer alone or with equilibrium buffer plus 10 mM methyl α-D-mannopyrannoside. Strongly bound material was eluted with 200 mM methyl α-D-mannopyrannoside.

SGP35 were examined by ConA-affinity chromatography (Fig. 4B). All the SNA-bound material applied to the column was recovered with 200 mM methyl-α-D-mannopyranose indicating high-mannose or hybrid glycopeptides [23].

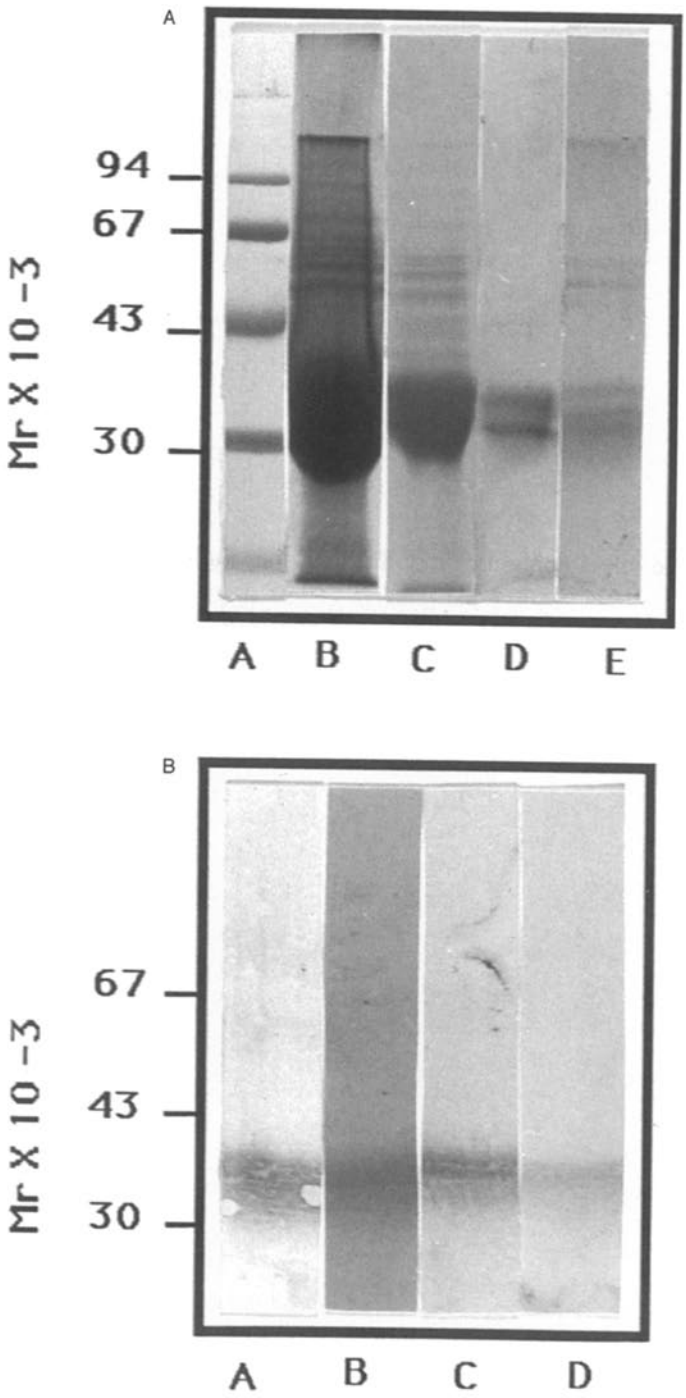

Figure 5. (A) SDS-PAGE of the protein at various steps of purification. Samples (10 μg) from each purification step were subjected to PAGE and the gels stained with Coomassie Blue. Lane B: detergent-soluble KC extract. Lane C: peak **I** from TSK-DEAE column. Lane D: bound material (fractions 21–42) from SNA-Sepharose column. Lane E: unbound material from SNA-Sepharose column (fractions 4–14). Mr values were estimated from molecular weight standards (lane A). (B) Lectin-reactivity of the carbohydrate chains of SGP35. Aliquots (15 μg) of SGP35 were separated by SDS-PAGE, transferred to nitrocellulose sheets and blotted with SNA (lane A), PHA-E (lane B), UEA (lane C) and WGA (lane D).

Table 1. AcPase activity.

	Proteins (mg)	*Total AcPase activity (nmol h^{-1})*	*Specific AcPase activity (nmol h^{-1} mg^{-1})*	*Purification*	*Recovery (%)*
Detergent-soluble KC extract (Cytosol 100 000 g)	9.2	195	21	1	100
Peak I from TSK-DEAE column	3.3	165	50	2.3	84
Bound material from SNA-Sepharose column (fractions 21–42)	0.1	130	1300	61	60
Unbound material from SNA-Sepharose column (fractions 4–12)	1.7	6	3	0.2	3

Enzymatic deglycosylation of the SGP35

Incubation of SGP35 with peptide-*N*-glycosidase F, which removed *N*-linked carbohydrate side chains [24], increased the electrophoretic mobility of the molecules (SGP35 proteins were smaller (33 kDa) following deglycosylation), and the protein bands became sharper. The electrophoretic resolution of the 36 and 34 kDa glycopeptides was poorer with sham-digested samples than with undigested samples (see Fig. 5A, lane D and Fig. 6, lane A). The electrophoretic shift between the sham-digested and the deglycosylated samples (lanes A and B in Fig. 6) indicate that, *N*-linked oligosaccharides account for about 3 and 1 kDa (8% and 3%) of the apparent molecular mass of the 36 and 34 kDa parent molecules, respectively. Deglycosylation also resulted in an effective decrease in the size microheterogeneity of the molecules, despite the general loss of resolution observed after digestion and sham-digestion. Amino-acid analysis of the deglycosylated SGP35 showed that the proteins contain 50% alanine, glutamic acid, tyrosine and lysine (Table 2).

LECTIN-REACTIVITY OF THE CARBOHYDRATE CHAINS OF SGP35

Oligosaccharide affinities of the two SGP35 glycopeptides were analysed by Western lectin-blotting. Both glycopeptides reacted with SNA and with ConA. The 36 kDa glycopeptide had a higher affinity for PHAE and UEA than did the 34 kDa. But the affinity of the 36 kDa unit for WGA was lower than for PHAE or UEA (Fig. 5B). Neither glycopeptide reacted with MAA, GNA, PNA or DSA (data not shown). Controls

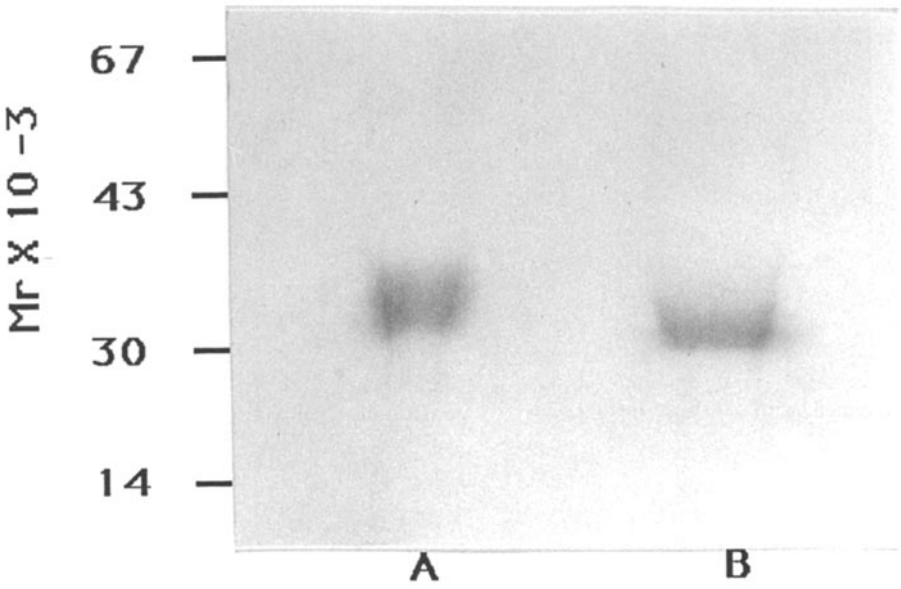

Figure 6. Deglycosylation of SGP35 by peptide-*N*-glycosidase F. Samples of SGP35 were denatured with 0.5% SDS and 0.1% Triton X100, incubated with PNGase F plus proteinase inhibitors, analysed by SDS (4–15%) gradient PAGE gel and stained with Coomassie Blue. Lane A: sham-digested SGP35, lane B: PNGase F-treated SGP35.

Table 2. Amino acid composition of SGP35.

	μg 100 μg^{-1} wt	*Residues per 100 amino acids*
Ala	8.8	15.5
Gly	1.8	3.6
Val	2.7	3.6
Thr	3.1	4.1
Ser	1.8	2.7
Leu	6.5	7.7
Ile	4.2	5.0
Pro	2.6	3.6
Aspx	3.0	3.6
Phe	8.1	7.7
Glux	9.4	10.0
Tyr	18.9	16.4
Lys	7.6	8.2
Arg	8.6	7.7

performed by dot-blotting on the SNA-unbound material showed the absence of SNA-reactive material from this fraction, while they revealed the presence of ConA-, PHAE- and UEA-reactive glycoprotein components (data not shown).

Discussion

At the light microscope level, in the thymus, a selective labelling of Kurloff cells by SNA was established [25]. At the ultrastructural level, the distribution of gold-labelled SNA on the KB, especially on myelin figures [11] and the distribution of AcPase activity [5, 6] were very similar. But the KC α2–6 sialoglycoconjugates are heterogeneous. They fall into two well-defined groups, SGP35 and SGP80, which differ in charge and mass. The SGP80 are anionic large molecules with a high sialic acid content. They undoubtedly contribute to the ultrastructural pattern of gold-SNA labelling seen on KC sections. The location of SGP80, which contain α2–8 polysialic acid residues (unpublished data), in KC is, as yet, unknown. These heavily sialylated molecules are absent from the weakly anionic fraction [13], and hence from SGP35. SGP35 and SGP80 were separated by anion-exchange chromatography. Thus, ion-exchange and SNA-affinity chromatography were used to purify SGP35. Their elution as two peaks, at 25 mM and 40 mM lactose, was reminiscent of the elution of disialylated and trisialylated fetuin molecules from SNA-Sepharose [26]. The 34 and 36 kDa glycoforms of SGP35 may also differ in the number of their sialic acid residues.

KC SGP35 produced two well-defined protein bands (34 and 36 kDa) by SDS-PAGE, and two main bands (190 kDa and 500 kDa) under non-denaturing conditions. This pattern was similar to the AcPase zymographic pattern [12]. There is also direct evidence for the AcPase activity of SGP35 and direct evidence that sialoAcPases are the major sialylated component of the KC. Both sialo- and asialo-AcPases of the KC were recovered from the fraction that was not bound to DEAE-cellulose, and they migrated at 35 kDa on SDS-PAGE.

Their size under reducing conditions and their location in the lysosome compartment suggest that KC sialoAcPases might be compared to the TRAP (type 5 AcPases), which are also glycoproteins with both sialo- and asialo-subunits (for review see [27]). However, TRAP and KC sialoAcPase differ in their pI, tartrate sensitivity and immunological properties (unpublished data). Since KC sialoAcPases are tartrate-inhibitable and lysosomal, they belong to AcPase type 2. To the best of our knowledge, the major structural features of type 2 AcPases in other leukocyte granules and particularly other NK cell granules have not been established. Progress in this domain will undoubtedly allow us to clarify the position of KC among leukocyte subsets.

Our data show that purified SGP35 possesses almost all the lectin reactivities previously observed in 30–35 kDa glycoproteins [12], with PHAE-, WGA-, ConA- and UEA-reactivities. The lack of GNA-, DSA-, MAA-reactivity in SGP35 was predicted by previous data obtained with crude-extracts. According to the specificities of these lectins [28, 29], our results indicate the absence of oligomannosidic type *N*-linked oligosaccharides, polylactosaminic sequences, and α2–3-linked sialic acid residues in SGP35. SGP35 did not react with PNA either before or after desialylation, indicating the absence of *O*-linked Galβ1–3GalNAc disaccharide sequences. The 36 kDa polypeptide contains a large amount of the PHAE- and UEA-reactive oligosaccharide sequences, and a lesser amount of WGA-reactive sequences. This suggests the presence of both Fuc(α1–6)GlcNAc(β1–N)AsN and GlcNAc(β1–4)Man(β1–4)GlcNAc(β1–4)GlcNAc(α1–N)AsN sequences. The latter bisected sequences seemed to account for both PHAE and WGA-reactivity, since sialidase treatment did not alter the WGA-reactivity of this material. However, these lectins might also interact with two distinct glycan chains. The presence of both UEA-reactivity and WGA-reactivity on the same glycoconjugate molecule strongly suggests that these two lectin-reactivities are borne by distinct *N*-linked oligosaccharides. An α1–6 fucosyl residue in the core has been reported to inhibit the interaction of the glycopeptide with WGA by steric hindrance [30]. The WGA-positive sequences of SGP35 may thus be borne by an oligosaccharide other than the UEA-positive one. According to Debray and Montreuil [30], the presence of both SNA-reactivity and PHAE-reactivity on the same oligosaccharide is very unlikely if the Gal residue of the Gal(β1–4)GlcNAc(β1–2)Man(α1–6) antenna is replaced by an α2–6-linked sialic acid. Under such conditions, the

SNA-positive sequences of SGP35 might also be borne by *N*-linked oligosaccharides distinct from the PHAE-positive one.

The abundance of bisecting GlcNAc residues and of α2–6 sialic acids residues in SGP35 probably reflects high activity of N-acetylglucosaminetransferase III (GlcNAc-T III, E.C. 2.4.144), responsible for adding the β1–4 GlcNAc to the trimannosyl core, and α2–6-sialyltransferase (α2–6 ST, E.C. 4.99.1).

The strong binding of SGP35 to Sepharose-ConA indicates the presence of the hybrid type of *N*-linked oligosaccharides on their peptide backbones as complex type chains would not be retained (tri-, tetra-antennary, either bisected or not, and bisected biantennary), or would only be weakly (non-bisected biantennary) retained on the column, while those of the high mannose type would react with GNA. Since fucosylated chains should not be bisected, there may be at least two types of oligosaccharide chain in sialoAcPase. Likewise, according to Schachter [31], the insertion of a bisecting GlcNAc prevents the actions of α-mannosidase II, core α6-fucosyltransferase, and GlcNAc-T II, IV and V, thereby effectively halting further branching. Therefore, the action of GlcNAc T III may have prevented the processing of hybrid to complex structures in KC. KC appear at puberty under oestrogen stimulation; the enzymes α2–6 ST and GlcNAc T III, that are responsible for the maturation of KB sialoglycoproteins, could also be regulated by oestrogens. The expression of α2–6 ST in hepatocytes is increased by corticoids [32], and by developmental factors.

The majority of the mammalian AcPases are glycoproteins with multiple glycoforms. Their heterogeneity is primarily due to variations in the carbohydrate residues, but the role of the carbohydrate chains has not been clarified. Previous studies suggested that intragranular AcPases are involved in the cytotoxic activity of the NK cells [33], and that α2–6-linked sialic acid residues may participate in NK cytolysis [15]. Finally, knowledge of the structure of the glycan moiety of KC sialoAcPase, which constitutes a quantitatively major component of the KB, would help to determine the physiological significance of KC. Moreover, the purified sialoAcPases of the KB described here may be a tool for developing specific anti-sialoAcPases antibodies and for determining specific inhibitors. Thus, their purification might constitute a milestone to ascertain the role of the KB and the role of its phosphatases in cytotoxicity.

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