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Preparative affinity electrophoresis of different glycoforms of serum glycoproteins: application for the study of inflammation-induced expression of sialyl-Lewis^x groups on α_1-acid glycoprotein (orosomucoid)

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During acute inflammation, human α_1-acid glycoprotein (AGP) is subject to marked changes in branching of its glycans, its degree of fucosylation and the expression of sialyl-Lewis^x (SLe^x) groups. To be able to study these changes in glycosylation in more detail, a procedure was developed to isolate the different glycoforms of AGP in milligram amounts by preparative affinity electrophoresis (AE) with a free lectin as fractionating agent. The method was applied to isolate differently fucosylated forms of AGP with the fucose-specific lectin *Aleuria aurantia* (AAL). AGP was separated into one non-reactive (A0) and four reactive (A1–A4) fractions. It was found that, in particular, the highly fucosylated fractions A3 and A4 contained the inflammation-induced SLe^x groups of AGP. Analysis by crossed affinoimmunoelectrophoresis (CAIE) with concanavalin A (Con A) of these different glycoforms of AGP showed that the presence of tri- and/or tetraantennary glycans, instead of diantennary glycans, was associated with a higher degree of fucosylation. Identical results were obtained by subjecting Con A-fractionated forms of AGP to CAIE with AAL as the affinocomponent. It is expected that this method of preparative AE can generally be applied to other glycoproteins, which can be separated in different glycoforms by CAIE using lectins.

Keywords: affinity electrophoresis, *Aleuria aurantia* lectin, concanavalin A, lectins, sialyl-Lewis^x

Introduction

α_1-Acid glycoprotein (AGP) is an acute phase glycoprotein containing five *N*-linked glycans [1] of the di-, tri- and/or tetraantennary type. During acute and chronic inflammation changes in glycosylation occur on human AGP [see 2 for recent review]. One of these changes is an increase in the amount of the concanavalin A (Con A)-most reactive forms of AGP [3–6]. These glycoforms have been shown to contain one or more diantennary glycans [7]. Another change in glycosylation was recently described by us [8], and concerns the increased degree of $\alpha 1 \rightarrow 3$ fucosylation. This was detected by changes in the affinity of AGP towards the fucose-specific *Aleuria aurantia* lectin (AAL) [9] in crossed affinoimmunoelectrophoresis (CAIE) [8]. Five glycoforms of AGP could be distinguished, differing in their AAL reactivity (A0: non reactive with AAL, A1–A4: increasingly reactive with AAL). In particular, the A3 and A4 forms are increased after laparotomy, severe burning and primary *sectio ceasario*. A constitutively elevated level of these two forms was detected in

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chronic inflammation (rheumatoid arthritis) relative to control sera [10]. Staining of AGP with the mouse monoclonal anti-sialyl-Lewis^x (SLe^x) IgM, CSLEX-1, on Western blots, showed that the increase in fucosylation during acute inflammation was accompanied by an elevated expression of SLe^x structures on AGP [8]. Although this was established for total AGP in serum it was not known which of the fucosylated AGP glycoforms contained the SLe^x groups.

Detailed knowledge of the oligosaccharide structures on each of the glycoforms under various inflammatory conditions is necessary to understand the regulation and biological function of the inflammation-induced changes in glycosylation. To that aim, milligram amounts of the different glycoforms of AGP are needed from sera of patients under various inflammatory conditions. Therefore, a method was developed employing the principle of affinity electrophoresis (AE) in the presence of a free lectin. This principle is superior to chromatography on Con A–Sepharose in that it achieves a complete separation of the various Con A-reactive forms of AGP [11] and also separates the various AAL-reactive forms of AGP (unpublished results). This method was applied to investigate on which of the AAL-reactive glycoforms (A1–A4) of human serum AGP, the acute-inflammation-induced expression of SLe^x occurred. Furthermore, the relationship between the degree of fucosylation of these fractions and the diantennary glycan content was studied under the inflammatory conditions.

Materials and methods

Materials

AGP was isolated by immunoaffinity chromatography from the serum of patients subjected to laparotomy and from a patient suffering from severe trauma as previously described [8]. Control AGP was isolated from Cohn fraction V supernatant of pooled human sera from healthy individuals according to Hao and Wickerhauser [12]. The purity of AGP was checked by SDS–PAGE followed by silver staining. *Aleuria aurantia* mushrooms were collected locally and AAL was isolated as detailed earlier [8]. α-Methyl D-mannoside agarose, L-fucose agarose, free Con A (Type V), methyl-α-D-glucopyranoside and α-L-fucose were purchased from Sigma (St Louis, MO). Rabbit anti-human AGP IgG was obtained from Dakopatts (Glostrup, Denmark); mouse anti-SLe^x IgM CSLEX-1 from Tissue Typing Laboratory, Department of Surgery, UCLA School of Medicine (Los Angeles, CA); and alkaline phosphatase-conjugated goat anti-mouse IgM from Zymed (San Fransisco, CA). All other materials used were of analytical grade and obtained from commercial sources.

Preparative affinity electrophoresis of α_1-acid glycoprotein

Fractionation of different glycoforms of AGP. The different glycoforms of purified AGP (1–6 mg) were separated by electrophoresis in a lectin-containing 1% agarose gel at 8 V/cm at 15°C for 2–6 h, depending on which lectin was used (Figure 1). The 1% agarose gel was prepared in 24.3 mM diethylphenobarbituric acid/Tris buffer (pH 8.6), containing 0.4 mM calcium lactate, 0.02% NaN_3 and 2 mg/ml Con A or 2.5 mg/ml of an AAL preparation (with a haemagglutination titer of 512) [8, 13]. The gel was cast using a glass plate provided with 5 mm thick PVC rims to make the gel 4 mm thick, 70 mm wide and 80 mm long. During the casting of the gel a PVC comb (70 mm × 3 mm × 3 mm) was placed at the application site for the preparation of a slot 3 mm deep (Figure 1). Care was taken that this slot did not protrude to the bottom of the glass plate to prevent insufficient separation and loss of protein by electrophoresis underneath the lectin gel. In order to determine the localization of the AGP fractions, a small test lane was taken from the middle of the gel, parallel to the direction of electrophoresis and subjected to crossed immunoelectrophoresis (10 V/cm, 2 h). To prevent diffusion during the test-lane analysis, the remainder of the gel was cut in portions of 1 cm perpendicular to the electrophoresis direction. The separated lanes were then stored in a wet chamber at 4°C, awaiting the recovery of the various glycoforms by electrophoresis (see below). When a lectin was used that had more than two reactive fractions, like AAL, the time of electrophoresis in the first gel was increased to obtain a better separation between the highly reactive fractions. This required an increased length of lectin-containing gel or the repeated (after 1–2 h of electrophoresis) removal of the anodal section of the gel (4 cm) and its replacement by a new lectin-containing gel.

Recovery in a DEAE–Sephacel-containing gel. To recover the various glycoforms each gel lane was subjected to electrophoresis (8 V/cm for 2 h at 15°C) in a new 4-mm thick, 80-mm wide agarose

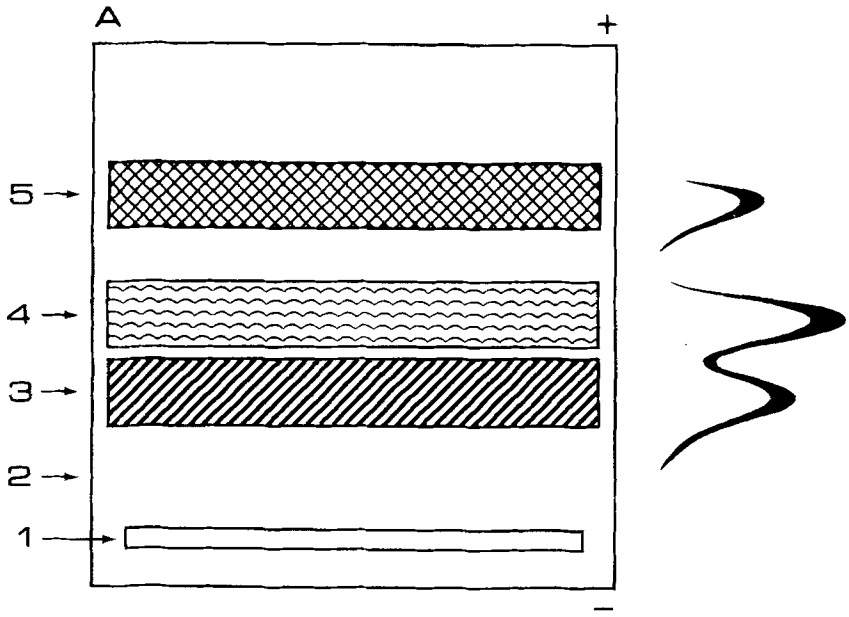

Figure 1. Procedure of preparative AE. Separation of the various AGP glycoforms was accomplished by electrophoresis in the lectin-containing gel of the preparative AE. The sample was applied in a 3 mm deep slot (1) in the lectin-containing gel (2). The fractionation of AGP by Con A is visualized by the hatched lanes. C2 is localized at position 3, C1 at position 4 and C0 at position 5.

gel in which (i) a specific eluting sugar was incorporated and (ii) a 2.2-ml DEAE–Sephacel suspension was present in the slot (4 mm × 7 mm × 80 mm). Methyl-α-D-glucopyranoside (0.1 M) was incorporated in the gel as eluting sugar; in the case of AAL, 0.01 M α-L-fucose was also incorporated. The DEAE–Sephacel-containing slot was prepared over the full width of the gel, perpendicular to the electrophoresis direction, in parallel to and at a distance of 5 mm from the glycoform-containing gel lane. The suspension of DEAE–Sephacel was prepared in electrophoresis buffer (DEAE–Sephacel/buffer ratio 3:1, v/v). The DEAE–Sephacel was collected and subsequently transferred to a small column (Pierce). The DEAE–Sephacel column was washed with 6 ml 0.1 M Tris/acetate (pH 8.6). Bound AGP was subsequently eluted with 6 ml phospate buffered saline (PBS) containing 0.3 M NaCl (pH 7.5). Fractions were desalted and concentrated by filtration over an Amicon PM10 filter at 55 psi. To check for purity of the fractions CAIE was performed according to Bøg-Hansen [8, 13]. For the removal of residual Con A and AAL the final fractions were subjected to chromatography on an α-methyl-D-mannoside-agarose column (1 ml) and L-fucose-agarose column (1 ml), respectively. The amount of AGP in each preparation was determined by radial immunodiffusion according to Mancini [14].

Further analytical methods

The 10% SDS–PAGE was performed according to Laemmli [15] using a Mini-Protean II dual slab gel apparatus (BioRad, Richmond, CA). Gels were loaded with the different forms of AGP, containing equal amounts of protein. Proteins were blotted onto nitrocellulose by electrophoretic transfer using a Mini Trans-Blot Cell (BioRad). SLe^x determinants on AGP were detected by incubating AGP-containing nitrocellulose strips with the mouse monoclonal anti-SLe^x IgM (CSLEX-1, 20 μg/ml in 10 times diluted PBS) [16], followed by alkaline phosphatase-conjugated goat anti-mouse IgM (1:250, v/v) for detection.

Results and discussion

Procedure of preparative AE

The procedure of AE as described in this study provides a method for the preparative isolation of different glycoforms of serum acute-phase glycoproteins in milligram amounts. The method was successfully applied in the isolation of the various AAL- and Con A-reactive fractions of AGP (Figures 2 and 3; Table 1), i.e, glycoforms of AGP that differ in the number of $\alpha 1 \rightarrow 3$-linked fucose residues and the number of diantennary glycans, respectively [1, 7–9, 17]. Since AGP contains five *N*-linked glycans [1], it is likely that the method will also be suitable for the isolation of different glycoforms of other, less abundantly glycosylated, acute-phase glycoproteins.

The CAIE patterns in the left panels of Figures 2 and 3 show that it is possible to obtain the different AAL- and Con A-reactive fractions of AGP, respectively. The small cross-contaminating fractions could be removed by resubjecting the

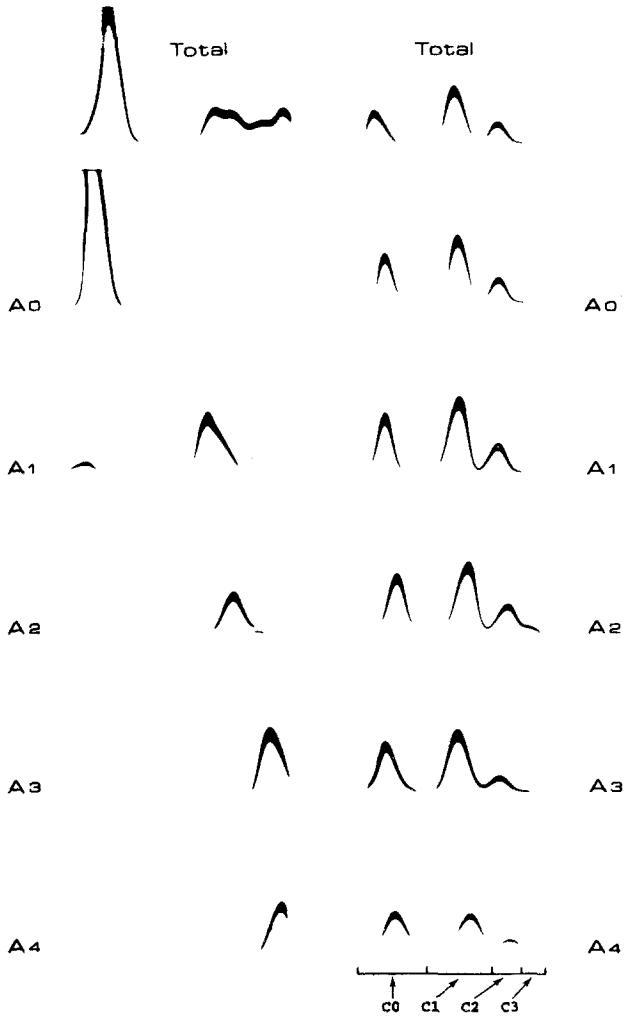

Figure 2. CAIE with AAL and Con A of AAL-fractionated AGP. AGP was isolated by immunoaffinity chromatography from the serum of patients subjected to laparotomy and was fractionated into a non-reactive (A0) and four reactive fractions with AAL (A1–A4) by preparative AE (left panel). CAIE with Con A as affinocomponent was performed with the different AAL fractions (right panel).

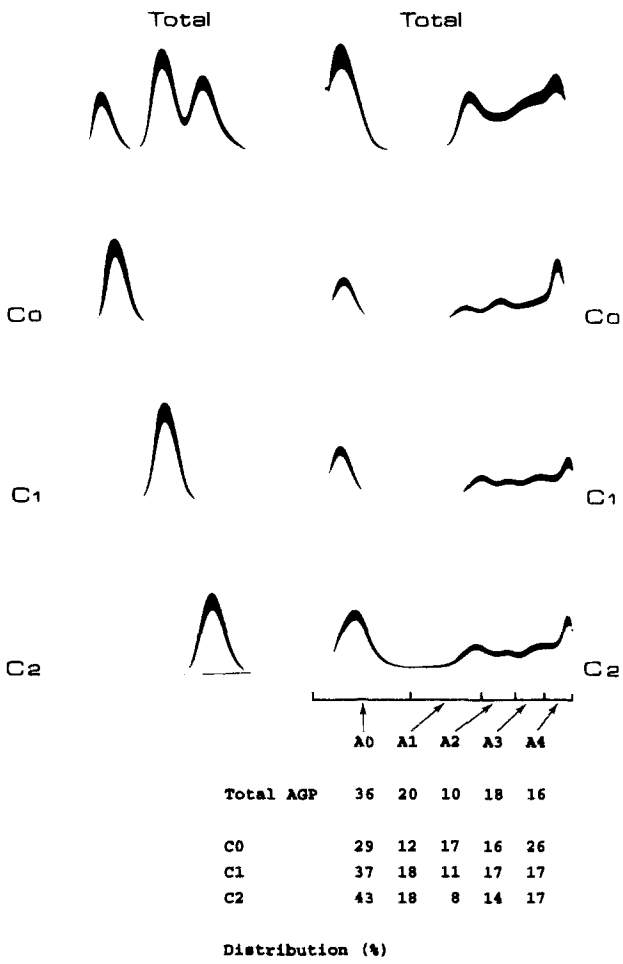

Figure 3. CAIE with Con A and AAL of Con A-fractionated AGP. Serum from a patient suffering from severe trauma was subjected to preparative AE and AGP was fractionated into a non-reactive (C0) and two reactive fractions with Con A (C1–C2) (left panel). CAIE with AAL as affinocomponent was performed with the different Con A fractions (right panel).

material to the AE procedure; this accomplished complete separation of the fractions (not shown). As is shown in Table 1 the relative recovery of the fractions corresponds very well with the CAIE patterns obtained with unfractionated AGP. Approximately 6 mg of AGP could be fractionated with a lectin-agarose gel of the given dimensions. This capacity can easily be increased by running several gels at the same time. An acceptable overall recovery was obtained when the different glycoforms of AGP were isolated by AE with Con A ($63 \pm 1.7\%$; Table 1), taking into account the number of steps of the total procedure. A lower recovery was obtained when AAL was used as the fractionating lectin ($41 \pm 7.5\%$; Table 1), which was most probably caused by a much stronger affinity of part of the AGP molecules for AAL than for Con A (see Table 1 and Figure 3). The

Table 1. Recovery of AGP in the fractions isolated by preparative AE with AAL (A0–A4) or with Con A (C0–C2)

AGP fraction	Overall recoveries[a]	Relative recoveries (%)	
		Determined[b]	Expected[c]
A0		65	57[d]
A1		14	13
A2		9	11
A3		7	8
A4		5	11
	41 ± 7.5 (n = 3)	100	100
C0		41	45
C1		51	47
C2		8	8
	63 ± 1.7 (n = 3)	100	100

Overall recoveries are given as means ± SD from three experiments (AE with total serum and purified AGP). Relative recoveries are given for a representative experiment.

[a]Corrected for losses in intermediate, overlapping fractions.

[b]As determined from the amount of AGP recovered in each fraction. The amount of AGP was determined by radial immunodiffusion and calculated relative to the amount of AGP (1–6 mg) applied to the lectin-containing gel.

[c]As expected from the relative surfaces of CAIE of total AGP.

[d]Inherent underestimation because peak extended into the non-linear area.

most retarded AGP molecules with Con A (C2) express only two binding sites for this lectin (di-antennary complex type glycans [7]), whereas the AAL-retarded AGP fractions A3 and A4 contain three or more binding sites for AAL, according to their electrophoretic mobility [see 13, 18 for a theoretical background of the CAIE method]. Changing the eluting or electrophoresis conditions in the recovery gel did not influence the recovery values.

The method does not necessarily require purified glycoproteins (Figure 2) as starting material, but can also be applied to total serum (Figure 3). This is of particular advantage for the further CAIE analyses of a lectin-fractionated acute-phase glycoprotein with other lectins. This is illustrated for serum AGP fractionated by Con A and subsequently subjected to CAIE analyses with AAL (Figure 3, right panel). An aliquot of serum (600 μl containing 1.9 mg AGP) from a trauma patient was subjected to AE with Con A. The AGP-containing parts of the gel were used in the recovery gel to prepare a non-reactive (C0) and two fractions reacting with Con A (C1 and C2) (Figure 3, left panel). These fractions were analysed further by CAIE with AAL (Figure 3, right panel).

Application of AE to study SLe^x substituted forms of AGP

Recently, we have described the acute-inflammation-induced expression of SLe^x on unfractionated AGP, which correlated with an increased reactivity of AGP with the fucose-specific lectin AAL [8]. The increase in SLe^x content of AGP was postulated to represent a humoral feed-back response on the SLe^x-E-selectin-mediated leukocyte–endothelial interaction, required for the extravasation of leukocytes into inflamed tissues. Under the acute-inflammatory conditions induced by laparotomy, the highly fucosylated fractions A3 and A4 of AGP, in particular, are increased relative to the level before surgery [8]. The method of AE was used to further investigate the relationship between SLe^x expression and AAL reactivity of AGP molecules. Using preparative AE, we clearly demonstrated that the expression of SLe^x structures occurs particularly on the fractions A3 and A4 (Figure 4a). No SLe^x expression was found on the non-fucosylated fraction A0, and only low expression was detected on A1 and A2. The low expression of SLe^x on control human serum AGP appeared to reside in fraction A4 and to a much lower extent in A3, whereas the expression in A1 and A2 was barely visible and no expression was

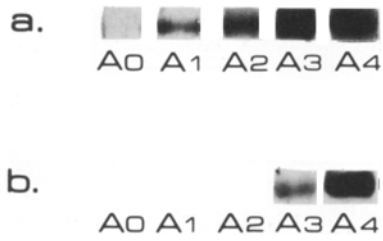

Figure 4. Detection of SLe^x on the various AAL-fractionated forms of AGP with the monoclonal IgM CSLEX-1. AAL-fractionated forms of AGP from patients subjected to laparotomy (a) and from healthy individuals (b) were stained with the monoclonal IgM CSLEX-1 to detect the expression of SLe^x groups. Each lane contained 3 μg of AGP.

found in A0 (Figure 4b). It can be concluded, therefore, that the observed correlation between SLe^x expression and AAL reactivity during acute inflammation [8] results from a strong increase in the highly fucosylated fractions A3 and A4. Furthermore, a comparison of the relative staining intensities of equal amounts of A1 and A2 with CSLEX-1 in control (Figure 4b) and inflamed (Figure 4a) conditions suggests that the degree of SLe^x expression increased also on these forms in inflammation. This observation, however, must be interpreted with care because all fucosylated fractions are heterogeneous with respect to their degree of branching, which in itself is subject to variations during inflammation [2–6]. Therefore, the glycosylation of the fractions was further studied by CAIE with the lectin Con A (see below).

Application of AE to study the relation between fucosylation and degree of branching of AGP

AGP from inflamed sera was subjected to fractionation with respect to fucosylation using AAL in AE followed by analyses of their degree of diantennary glycan content by CAIE with Con A (Figure 2) and *vice versa* (Figure 3). In Figure 2 it can be seen that the fractions A3 and A4 consisted of a higher amount of C0 molecules than the fraction A0, *i.e.* A3 and A4 contained 37 and 44%, respectively, of the C0 molecules, whereas A0 contained 30% of the C0 molecules. Figure 3 shows that the fraction C0 contained 29% of the A0 and 12% of the fraction A1. These percentages are lower than for fraction C2, which contained 43% of A0 and 18% of A1. These data show that the presence of tri- and/or tetraantennary glycans on AGP molecules rather than diantennary glycans coincides with a higher degree of fucosylation. The same observations, though less pronounced, were found for Con A fractions and AAL fractions of pooled human serum from healthy individuals (unpublished results). These results suggest that fucose residues on the glycans of AGP are present on tri- and tetraantennae in particular, which is in agreement with results of structural studies [17, 19–26]. This preferential occurrence of α3-linked fucose ($Lewis^x$; Le^x) and SLe^x is consistent with the specificity of the glycosyltransferases involved in their synthesis in relation to the activity of competing α6-sialyltransferase. The latter enzyme forms the major sialyltransferase activity of human hepatocytes [27] (the cells which produce AGP), and shows a decreased ability to sialylate *N*-linked glycans with a higher degree of branching [28]. By contrast, α3-sialyltransferase and α3-fucosyltransferase show a slightly elevated activity with oligosaccharides that contain an additional $1 \rightarrow 4/1 \rightarrow 3$ branch, as is the case in the tri- and tetraantennary glycans on AGP [29–31]. In addition, the action of α6-sialyltransferase is mutually exclusive with those of the α3-sialyltransferase and the α3-fucosyltransferase [32]. Therefore, increased branching of the acceptor glycan will result in a shift from branch termination by an α6-linked neuraminic acid (NeuAc) (i) to termination by an α3-linked NeuAc, (ii) by substitution of an α3-linked fucose to give the Le^x determinant or (iii) by substitution with both these residues to yield the SLe^x structure. It can be assumed that the increased SLe^x content of AGP during acute inflammation is determined by the same rules, *i.e.* by the relative activities of the three glycosyltransferases under this pathological condition. The acute-phase induced reduction of the activity of the α6-sialyltransferase (caused by mislocalization in and/or an increased release from the Golgi apparatus [33]) will reinforce the increase in SLe^x by changing the sialylation from $\alpha 2 \rightarrow 6$- to $\alpha 2 \rightarrow 3$-linked NeuAc. In order to determine the actual branch localization of SLe^x-determinants, further analytical investigations must be performed.

The method of AE has made these studies possible. It is expected that the method of preparative AE can generally be applied to other glycoproteins, which can be separated in different glycoforms by CAIE using lectins. Studies of the specific glycosylation on the different glycoforms of these glycoproteins can then be performed in more detail.

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