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Review

Lectin affinity immunoelectrophoresis of serum glycoproteins

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Abstract

The basic methodology of two major affinity immunoelectrophoresis techniques, i.e., crossed affinity immuno-electrophoresis and lectin affinity electrophoresis with immunoblotting, is described. This review covers the use of these procedures in the study of the mechanisms regulating protein glycosylation of several serum proteins from humans and animals. Applications of these techniques in fields of biochemical and clinical interest, such as inflammation, cancer, pregnancy and foetal development, are discussed.

Contents

1. Introduction

Affinity electrophoresis refers to any technique in which two or more components interact

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specifically during electrophoresis [1]. These techniques have been applied to many systems and particularly to serum proteins interacting with lectins. After the electrophoresis the components are often revealed by specific immunochemical methods. In this case, the techniques

should be named affinoimmunoelectrophoresis (AIE). Several reviews that covered the principles and general applications of the affinity electrophoresis techniques have been published [1–5].

Most serum proteins contain one or more oligosaccharide glycans attached to O-(serine/threonine) or N-(asparagine) glycosylation sites in their polypeptide backbone [6,7]. The glycan structures of numerous isolated plasma proteins have been determined [6]. The N-glycan structures more frequently found in mammalian serum proteins [6] are summarized in Fig. 1. Major glycan heterogeneity [7] depends on the number of N-acetyl lactosaminyl substitutions in the peripheral mannoses (bi-, tri- or tetra-antennary glycans principally), and on the presence or not of bisected N-acetylglucosamine (GlcNAc) bound to the central mannose. Changes in sialic acid (NeuAc) or fucose (Fuc) content produce the so-called minor heterogeneity [7]. The glycans of serum proteins could present both major and minor heterogeneities. Therefore, the number of glycoforms for a particular protein increases greatly with increasing number of glycans on its molecule. The heterogeneity found in the glycan moiety of serum proteins is the result of the action of many glycosylation enzymes, whose activities depend on the type and the physiological state of the cells synthesizing the serum proteins [8,9]. Homologous serum proteins from different species often possess glycans with similar structures [5,6], although for some proteins, such as α-fetoprotein (AFP) and α_1-acid glycoprotein (AGP), the number of glycans per molecule can be different [10–12].

The analysis of the complex-type glycans (N-glycans) of serum proteins and other glycoproteins is difficult and requires previous isolation of the protein and the use of sophisticated chemical and instrumental techniques [13]. However, a partial, although significant, characterization of the glycan structures can be easily obtained using lectin affinity chromatography or even better using AIE. With these methods, the glycoproteins could be directly determined in complex mixtures, such as blood serum, without being isolated. The serum protein glycoforms fractionated using affinity electrophoresis can be revealed and quantified with specific reactives, such as antibodies. As there are available lectins, i.e., sugar-binding proteins, which can recognize specifically most of the differences in the glycan structures [14], AIE can be applied almost without restriction to the analysis of serum protein mixtures.

Two major techniques will be discussed here in relation to serum proteins: (a) crossed affinity immunoelectrophoresis (CAIE), which uses a second-dimension immunoelectrophoresis to identify single proteins [15,16], and (b) lectin affinity electrophoresis (LAE) combined with antibody-affinity blotting to detect the fractionated proteins [17]. AIE has been applied to the determination of several proteins from human serum and in sera of other species, both in normal and in pathological conditions. We centre the review on recent results and on the general applications of AIE to the determination of serum proteins in physiological and pathological states.
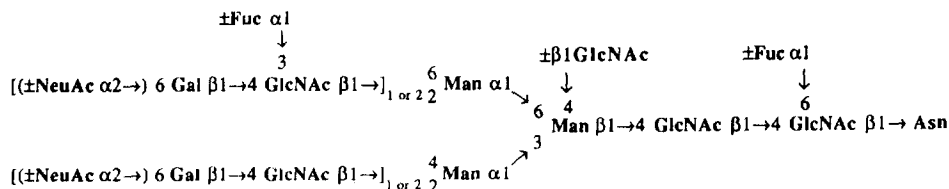

Fig. 1. More frequent N-glycan structures found in serum proteins. Major heterogeneity referred to the number of antennae (in brackets). Variations in Fuc and NeuAc add further heterogeneity.

2. Affinoimmunoelectrophoresis (AIE): principles and methodology

2.1. Lectins used in AIE

Lectins represent a key tool in AIE. There is considerable information about the biochemical properties of numerous lectins and the type of glycan structure that they can recognize [14,18, 19]. The lectins can be used in AIE of serum proteins when their pI is near to the alkaline pH (around 8.2–8.6) that is often used in electrophoresis [5]. This condition is achieved by numerous lectins, but in practice only a few of them have been systematically used in AIE. Concanavalin A (Con A), the first and the most often used lectin in AIE (ConA-CAIE), recognizes mainly biantennary glycans (Fig. 1) and oligomannosidic-type glycans (uncommon). Tri- or polyantennary glycans or $\beta 1 \rightarrow 4$ GlcNAc bisected glycans (Fig. 1) are not recognized by the lectin. The interaction of Con A with serum proteins becomes stronger as the number of biantennary glycans in the protein increases [16]. *Lens culinaris* agglutinin (LCA) or lentin lectin interacts mainly with bi-, GlcNAc bisected and some triantennary complex-type glycans possessing $\alpha 1 \rightarrow 6$ L-Fuc linked to the innermost GlcNAc (that are bound to N-asparagine, Fig. 1). *Phaseolus vulgaris* isolectin E-4 (E-PHA) recognizes GlcNAc bisected biantennary glycans or GlcNAc bisected triantennary glycans with terminal galactose (Gal). *Aleuria aurantia* agglutinin (AAA) is a Fuc-binding lectin, which preferentially binds to complex-type glycans with Fuc in an $\alpha 1 \rightarrow 6$ linkage to the innermost GlcNAc. Additional Fuc, $\alpha 1 \rightarrow 3$ linked, enhances the affinity of AAA for these glycans. This lectin is particularly useful to discriminate different L-Fuc-substituted glycans. *Pisum sativum* agglutinin (PSA) is a good tool to distinguish $\alpha 1 \rightarrow 6$ Fuc-substituted biantennary glycans from other fucosylated derivatives. Wheat germ agglutinin (WGA) interacts mainly with bisected biantennary complex-type glycans (Fig. 1) and with other less frequent bisected hybrid-type glycans with a polymannose bound to the core β-mannose. For other lectins used in

AIE and for a more detailed description of the glycan specificity of the lectins above mentioned, extensive reviews are available [14,18,19].

2.2. Crossed affinity immunoelectrophoresis (CAIE)

CAIE was first described by Bøg-Hansen et al. [15,16] and, although other technical modifications have been developed since then [5], the original description [16], with minor modifications, is the most often used method. The serum proteins are separated by electrophoresis in agarose gels containing an appropriate amount of a lectin, solubilized either in Tris–veronal-calcium lactate buffer (pH 8.2–8.6) or other comparable buffers (first-dimension gel). Under these experimental conditions most serum proteins migrate to the anode whereas the lectins have no mobility. During this first electrophoretic run, the proteins are fractionated into different microforms depending on the affinity degree of each microform for the lectin. Prolonged electrophoresis is necessary for the analysis of serum proteins with low electrophoretic mobility, such as transferrin. Alternatively, this type of protein should be chemically modified, for instance by succinylation or carbamylation [20]. Then the separated components are run in a second-dimension agarose gel, of moderate electroendosmosis, containing antibodies against the proteins to be analysed. In this second-dimension gel an appropriate amount of soluble oligosaccharide that competes with the lectin should be included, e.g., 50–100 mM methyl α-D-glucopyranoside for Con A [21] or 10 mM Fuc for AAA [9]. Alternatively, the gel containing the antiserum can be separated from the first-dimension gel by an intermediate gel (about 1 cm wide) containing the competitor oligosaccharide [5]. As a result of the electroendosmotic effect the competitor oligosaccharide moves into the first-dimension gel and favours the dissociation of the glycoprotein–lectin complexes and the redissolution of the glycoprotein–lectin precipitates. These precipitates are frequently observed when sera or other complex samples are used in the first electrophoresis. In addition, the

specific oligosaccharide displacer allows the detection of glycoforms, which are undetectable or underestimated without the displacer [21,22]. Artifactual peaks of low electrophoretic mobility could be generated owing to ill-defined precipitates formed in the first-dimension gel [1,22,23]. These artifactual peaks, which are probably produced by coprecipitation and entrapment phenomena, could be eliminated either by modifying the glycoprotein to lectin ratio during the first electrophoresis [22,23] or by the addition of the oligosaccharide competitor in the application well before the first electrophoretic run [23]. Good results can be obtained if the CAIE is performed with the purified glycoproteins [5].

The analysis of many serum glycoproteins revealed the formation of fused precipitation peaks (microforms) in the second-dimension gel. The amount of each microform is related to the corresponding immunoprecipitation area. This can be calculated by planimetry or by triangulation [5]. Image-processing systems allow an easy and more accurate determination of precipitation peaks [24]. It is always advisable to perform a parallel run with the sample in the absence of the lectin, because differences in the sialic acid content can modify per se the mobility of the glycoprotein [25,26]. A practical parameter to compare the pattern of microforms is the ratio between the sum of the areas of the reactive forms to that of non-reactive forms, defined as the reactivity coefficient (RC) [27].

In general, the patterns of microforms obtained by CAIE or by lectin affinity chromatography are similar, although higher resolution is obtained by CAIE [26,28]. A variant of CAIE used on a semi-preparative scale should be preferable to affinity chromatography for the isolation of glycoforms [29].

As an example of CAIE, the Con A patterns and their crossed immunoelectrophoresis controls (without Con A) for two serum proteins, transferrin (Tf) and α_1-antitrypsin (PI) from foetal pig, are shown in Fig. 2. PI shows a multi-peak pattern (Fig. 2B). The different peaks have been labelled in decreasing order of mobility [30]. This form of designation will be
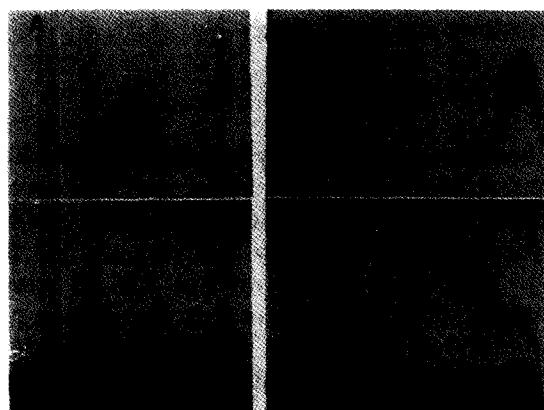

Fig. 2. Con A-CAIE patterns of (A) Tf and (B) PI from foetal pig serum. Top, control without lectin; bottom, with Con A. The microforms are numbered in decreasing order of mobility. First electrophoretic run from left to right.

used later for other glycoproteins. The first peak (C1) is the microform that did not react with the lectin. The other three microforms are weakly reactive (C2), reactive (C3) and strongly reactive (C4) with the lectin. In contrast, Tf shows a single component, which corresponds to a microform reactive against the lectin (Fig. 2A).

The CAIE patterns, such as those in Fig. 2, should be made visible after staining of the immunoprecipitates with Coomassie Blue or other protein dyes, which have a relatively low sensitivity. This is not a limitation for the major serum proteins. With some proteins, such as AFP, whose concentration in serum or in other biological fluids is often lower than 1 μg/ml [31], CAIE can only be performed if more sensitive techniques are used to detect the protein after the first-dimension gel. The use of radiolabelled proteins as markers [32,33] or immunoenzymatic methods [34,35], although more time consuming, provides the sensitivity required.

2.3. Lectin affinity electrophoresis (LAE) with immunoblotting

LAE combined with immunoblotting, which allows the analysis of human AFP glycoforms with high resolution and sensitivity, has been described [17,36,37]. In this technique the mi-

croforms of human AFP were first separated by electrophoresis in lectin-containing agarose gels, then the separated microforms were transferred to nitrocellulose membranes containing equine antibodies against AFP. After incubation of the membranes, the protein was revealed first with rabbit antiserum to AFP, and then with a second antibody, goat anti-rabbit immunoglobulin G (IgG), labelled with horseradish peroxidase. With this technique, microforms of AFP in samples at a concentration of 2 ng/ml or less can be easily determined. Fig. 3 shows the application of this method to the analysis, against several lectins, of human AFP [31]. Protein samples were collected from cord serum and from sera of patients with different pathologies. The number of microforms detected varied from two, with Con A, to five in the presence of E-PHA or *Datura stramonium* agglutinin (DSA). It is possible to discriminate with almost total confidence the different pathologies comparing the patterns obtained with each lectin [31,38,39]. The results obtained by LAE with immunoblotting and by CAIE are similar for the same concentration of human AFP [40]. Although this

type of LAE has been applied to the study of human AFP microforms, it might be used to analyse the microheterogeneity of other low-concentration serum proteins, e.g., Tf produced by cultured cell lines [41]. In addition, two-dimensional LAE, using different lectins in each dimension, might provide a lot of information about the glycan heterogeneity of serum proteins [42,43].

3. Determination of serum proteins by AIE

3.1. α-Fetoprotein (AFP)

AFP is a major component in the serum of foetal and neonatal mammals [44,45]. This protein is synthesized in early embryos by the yolk sac and then by the foetal liver, principally [44]. Later the expression of the AFP gene is repressed and the protein disappears from circulation. The concentration of AFP reported in healthy human adults is always lower than 6 ng/ml [31]. An increased serum concentration of AFP in human patients is associated mainly with
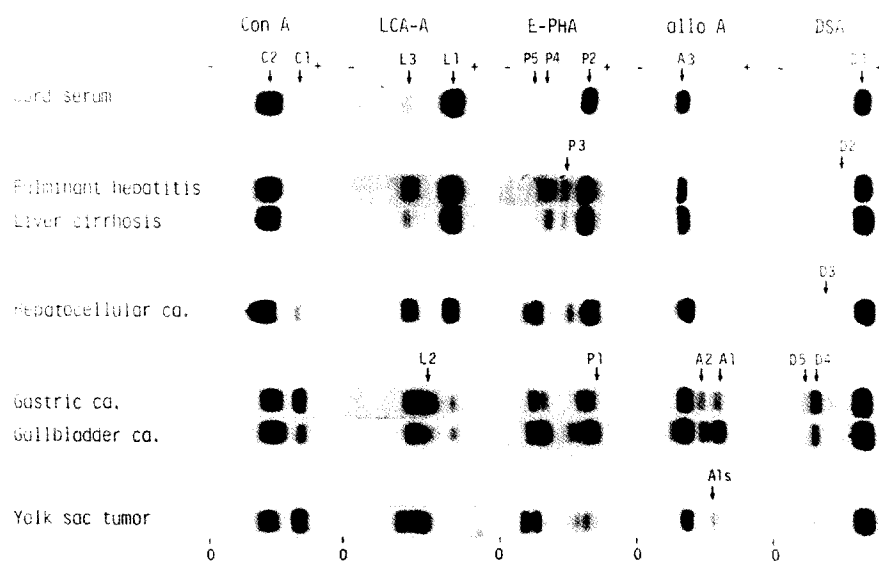
Fig. 3. Representative pattern of AFP glycoforms obtained by LAE, with different lectins and combined antibody-affinity blotting, for benign and malignant diseases. From Ref. [31].

embryonic cancers, hepatocellular carcinoma and other hepatic pathologies such as liver cirrhosis, chronic hepatitis and hepatitis B [31]. Changes in the normal concentration of human AFP in maternal serum and amniotic fluid, during foetal development, are related to neural tube defects [46].

The physico-chemical and biochemical properties of AFP have been extensively studied [45]. Human AFP is a single-chain polypeptide with $M_r \approx 70\,000$, containing one asparagine-linked glycan [47]. Bovine and rat AFP possess two of these glycans [10,11]. The physiological function of this protein is still unknown. AFP, as albumin, can transport fatty acids, preferentially arachidonic and docosahexaenoic fatty acids [48–50]. These fatty acids are characteristic components of the phospholipids from neural tissues and they accumulate there during ontogenesis. Therefore, AFP may be the physiological carrier of these fatty acids during development [50].

AFP shows high heterogeneity in its molecule owing to the ligand content [48] and to the glycan composition. Glycan heterogeneity can change greatly depending on the species and their physiopathological states. The glycan of human AFP from foetal serum [51] or produced by hepatomas [52] is mainly biantennary; a high proportion of GlcNAc bisecting glycan appears in AFP from yolk sac tumours [47]. Additional heterogeneity is produced by $\alpha 1 \to 6$-linked Fuc to innermost GlcNAc and by the content of sialic acids [43,51,52]. The structure of the two glycans in rat AFP is similar to that in human AFP [10]. In bovine AFP the glycans with triantennary structure predominate [11].

The glycan heterogeneity of AFP can be revealed by AIE [31,46]. Fig. 4A shows a typical Con A-CAIE pattern of AFP from human amniotic fluid. The major component (93%), which interacts with the lectin, is accounted for by AFP variants with biantennary glycans [51,52]. The minor component corresponds to AFP with GlcNAc bisecting biantennary glycans [47,52]. The pattern of AFP from foetal pig serum is very similar to that of human AFP from amniotic fluid (Fig. 4B). The pattern of rat AFP from neonatal serum shows three components (Fig. 4C), which, in ascending order of mobility, correspond to AFP variants with two biantennary glycans (strongly reactive component), with one biantennary glycan (weakly reactive component) and with two bisected biantennary glycans (non-reactive component), respectively [10]. A similar pattern has been reported for mouse AFP [53,54].

The CAIE pattern of AFP using other lectins is different [53,54]. With LCA the CAIE pattern for human AFP shows three components, strongly, weakly and non-reactive forms [53–55]. The glycans of the strongly reactive fraction have Fuc bound to the first GlcNAc [56]. The fraction weakly reactive is also Con A non-reactive [36] and, therefore, might correspond to glycans containing bisecting GlcNAc [31]. The non-reactive fraction corresponds to AFP with non-fucosylated glycans. The rat and mouse AFP also showed three microforms using CAIE with LCA [53,54]. Using CAIE with E-PHA human AFP might present as many as five different microforms depending on the origin of the sample analysed [57].

As indicated previously, CAIE can be routinely applied to samples containing AFP at con-

Fig. 4. Con A-CAIE patterns of AFP from (A) human amniotic fluid, (B) foetal pig serum and (C) neonatal rat serum.

centrations greater than 1 μg/ml. These concentrations are easily found in human umbilical cord serum and in sera from some cancer patients [31]. However, for most cancer patients, and also for patients with other hepatic pathologies (cirrhosis, chronic hepatitis and hepatitis B) or in serum and amniotic fluid from pregnant women, the typical AFP concentrations ranged between 20 ng/ml and less than 1 μg/ml (reviewed in Ref. [31]). As we shall discuss later, the analysis of AFP glycan heterogeneity could be of greater clinical interest. This is centred on the establishment of diagnosis criteria to distinguish different pathological situations. For example, CAIE has been used to discriminate between hepatocarcinoma from other less harmful hepatic diseases or between normal foetuses and those with neural tube defects. LAE combined with immunoblotting [17,36] can be applied to differentiate the several pathologies mentioned above, as illustrated in Fig. 3. Among the lectins used in this technique, E-PHA might add particularly useful information in the analysis of AFP glycoforms. The AFP-P4 microform is characteristic of hepatocellular carcinomas and AFP-P5 increases in yolk sac tumours (Fig. 3). The glycan structure of these and other micro forms of human AFP detected by E-PHA have been determined recently [43,58].

3.2. α₁-Acid glycoprotein (AGP)

AGP (orosomucoid) is a highly glycosylated serum protein in mammals whose concentration varies greatly depending on the species and the physiopathological states [59,60]. The protein

from human serum has been extensively studied. The human protein consists of a single polypeptide chain of 181 amino acids and five N-linked glycans [12,59]. AGP shows sequence homology with other serum proteins, especially with immunoglobulins [61] and with epidermal growth factor [62]. AGP has been characterized as an acute phase protein, because for human and other species its serum concentration increases substantially in response to infection and inflammation [60]. However, in pigs the serum concentration of AGP did not increase acutely after experimentally induced inflammation [63]. The biological role of AGP is still unknown, although some immunoregulatory properties for this protein have been suggested [9,64].

AGP shows multiple heterogeneity: its polypeptide chain presents genetic isoforms with several amino acid substitutions and numerous variations in the structure of their constitutive glycans [59,65]. All genetic forms of AGP present similar glycosylation patterns [65]. The glycan heterogeneity of AGP from human and rat, which possesses six N-linked glycans [12], is mainly due to the number of antennae (two, three and four) in each glycan and also to the Fuc and sialic acid content, as occurred in other glycoproteins [66,67].

AGP has been largely studied by Con A-CAIE. Fig. 5 illustrates the Con A-CAIE patterns of AGP from (A) normal human, (B) pig and (C) rat sera. The proteins from human, rat, pig and bovine species present a similar glycosylation pattern, with three or four components [67–72]. The microform with higher electrophoretic mobility contains tri- or tetra-
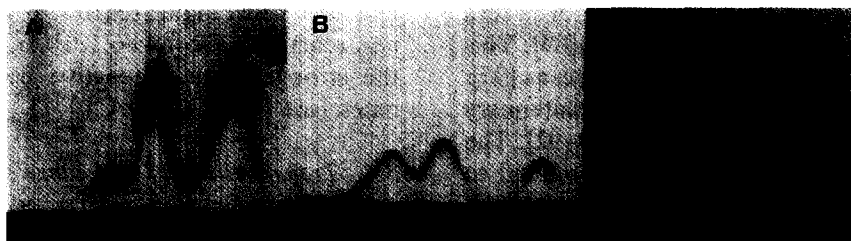

Fig. 5. Con A-CAIE patterns of (A) human AGP from serum of healthy individuals, (B) isolated AGP from normal adult pigs and (C) AGP from normal adult rats.

antennary glycans [66,73]. However, this microform might possibly possess biantennary glycans with $\alpha 2 \rightarrow 6$-linked sialic acids that do not react with the lectin. This structure has been observed in rat AGP [73]. The other fractions correspond to microforms with one or more biantennary chains [66]. The use of AAA in the analysis of human AGP by CAIE allows the detection of glycans with a sialyl Lewis-X structure, which increase significantly in inflammatory processes [9].

The microheterogeneity patterns of AGP, analysed by CAIE, are altered in diverse physiological and pathological states. An increased proportion of the Con A-reactive AGP microforms has been observed, for example, in patients with acute inflammatory diseases, burns, surgical trauma and acute infection [68,69,74–77]. These microforms may also increase in AGP from patients with different tumours [75,78]. On the other hand, a decrease in the Con A reactivity of AGP is found in patients with liver cirrhosis, severe rheumatoid arthritis, ankylosing spondylitis and chronic bacterial infections, and also in estrogen treatments, pregnancy and some tumours [76,78–80]. As discussed later, the analysis of AGP by CAIE is useful for clinical diagnosis and management of patients with inflammatory processes and perhaps in some cancers [77,78,81,82]. In addition the increasing clinical application of the analysis of AGP by CAIE, this technique has been used to study the effect of hormones and cytokines on the mechanisms that regulate the glycosylation of this and other serum proteins [83–88].

3.3. α_1-Antitrypsin (PI) and α_2-HS glycoprotein

PI is a major serine proteinase inhibitor in sera from human and other animal species [89]. This protein contains three complex-type glycans [90]. These glycans possess biantennary, triantennary and bisected biantennary structures [90–92]. The physiological function of PI could be related to its capacity for inhibiting serine proteinases [89]. Individuals with severe deficiency in PI develop pulmonary emphysema at an early age [89]. In human and rat, PI is a moderate acute-phase

protein [60], but this protein did not increase in pigs with acute inflammation [63].

By Con A-CAIE, PI exhibits high heterogeneity. Human PI shows a pattern containing from three to five components [25,27,69,84, 85,93,94]. The Con A weakly reactive fraction clearly predominates in normal serum [69,94]. The PI produced by cultured hepatocytes and hepatoma cells presents a different CAIE pattern, particularly in the presence of cytokines which stimulate the synthesis of acute-phase proteins [27,84,93]. A representative Con A-CAIE pattern of pig PI is shown in Fig. 2B. This protein may contain four microforms, predominating the Con A-reactive forms [71]. In the pattern of rat PI, with four components, the reactive glycoforms also predominate [67,95]. The glycosylation of human PI has been studied in the serum of patients with different diseases, although the changes observed are of lower intensity than for AGP [69,94].

α_2-HS glycoprotein is a protein from human serum with $M_r \approx 49\,000$. It has been reported to be implicated in a number of physiological functions such as brain and bone tissue development [96]. It is known that this protein is the human analogue of bovine fetuin [97], a protease inhibitor found in high concentration in serum from bovine foetuses and also in other foetal ruminants and pigs [98]. Fetuin possesses three N-glycans and three O-glycans [99]. Although the function of fetuin is ill-defined, it has been reported that a phosphorylated form of fetuin may inhibit the activation of insulin receptor [100]. α_2-HS glycoprotein and its homologous pig fetuin may be considered as negative acute-phase proteins [63,96].

Using CAIE against Con A, α_2-HS glycoprotein shows two major reactive peaks and a minor non-reactive peak [69,86,101]. In adult pig fetuin the stronger Con A-reactive fraction predominates (see Fig. 8A and C) [71].

3.4. Transferrin (Tf) and other serum proteins

Tf is an iron-binding protein with $M_r \approx 79\,000$ and constituted by two N-linked glycans [102]. In normal serum the glycans of Tf are mainly bi- or

triantennary fully sialylated and without Fuc [103,104]. Combinations of these glycan types can also be observed in the Tf microforms [104]. Tf is required for all proliferating cells in vitro and in vivo [105] and is synthesized mainly by the liver [105], although synthesis of Tf by other cell types has been described [106].

The analysis of human Tf by Con A-CAIE results in the formation of three peaks, which correspond, according to the ascending order of mobility in the gel, to microforms containing two, one or no biantennary glycans, respectively [23,24,104,107]. Frequently an artifactual peak is produced near the application well, but it might be avoided [23,24]. Changes in the Con A-CAIE pattern of human Tf have been observed, for example, in alcoholics [24], in pregnancy [104] and in the Tf produced by hepatomas [107].

α_1-Antichymotrypsin is a highly glycosylated protein, characteristic of human serum. This protein inhibits chymotrypsin-like proteases [89] and its serum concentration increases during the acute phase [60]. Using Con A-CAIE, α_1-antichymotrypsin from normal serum, which contains four glycans [108], should present three components in about the same proportion [69,108,109]. The microforms interacting with Con A, as occurred for other serum proteins, increase in acute inflammatory pathologies [69,108].

In addition to the proteins described above, affinity electrophoretic methods have been used in the analysis of other serum proteins: haptoglobin [110], ceruloplasmin [27], antithrombin III [111], pregnancy-associated plasma protein-A [112], prothrombin [113], serum cholinesterase [114] and serum alkaline phosphatase [115].

4. Biomedical applications of AIE

AIE has been applied to the analysis of the microheterogeneity of several serum glycoproteins in many pathological states. Therefore, in the last few years a lot of information has been obtained about the relationship between changes in the microheterogeneity and the evolution of diseases. Moreover, AIE has been used as a simple tool to understand better the mechanisms implicated in the glycosylation of serum proteins. In this section we comment on the most relevant fields in which AIE of serum proteins has been applied: inflammation, cancer, pregnancy and foetal development. As an example, Fig. 6 shows the Con A-CAIE analysis of human AGP in (A) inflammatory processes, (B) umbilical cord and (C) hepatoma ascites.

4.1. Inflammation

In response to tissue injury and infection, the liver greatly modifies the synthesis of serum proteins. Acute-phase proteins are those proteins whose concentrations in the serum increase greatly as a result of these processes [60]. C-reactive protein, serum amyloid A, AGP, PI, haptoglobin, α_1-antichymotrypsin, ceruloplasmin and fibrinogen, among others, are acute-phase serum proteins in humans. The serum concen-
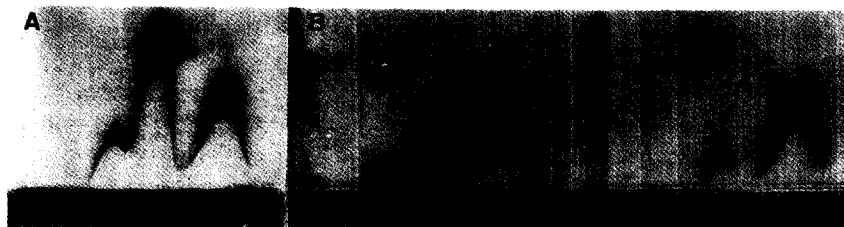

Fig. 6. Con A-CAIE patterns of human AGP from (A) serum of patients with inflammatory processes, (B) umbilical cord serum and (C) ascites of patient with a hepatocarcinoma.

trations of albumin and α_2-HS glycoprotein, on the other hand, decrease during inflammation and therefore they are considered as negative acute-phase proteins [60]. The synthesis of acute-phase proteins by hepatocytes and hepatomas could be induced by glucocorticoids and by cytokines, principally interleukin-1 (IL-1), tumour necrosis factor (TNF) and interleukin-6 (IL-6). These cytokines are released by monocytes and other cells in the early phase of inflammation [116,117]. Most of the acute-phase proteins are glycosylated and the pattern of glycosylation also changes during inflammation. Early studies using Con A-CAIE showed increases in the biantennary microforms of AGP from patients with acute inflammation, as defined by high levels of C-reactive protein in serum. A characteristic Con A-CAIE pattern of AGP during inflammation is shown in Fig. 6A (control in Fig. 5A). Similar changes were observed in α_1-antichymotrypsin, PI, α_2-HS glycoprotein and ceruloplasmin [68,69]. Moreover, CAIE analyses have been carried out for proteins in serum from animals with induced experimental inflammation [26,65,67,83,87,88,118] and in culture media from normal or tumoural cells [26,27,65,67,83,84,86,93]. The results obtained in these experimental models were similar to those observed in human patients. Fig. 7 shows the glycosylation patterns of AGP, using Con A-CAIE, from rats injected with (A) complete Freund's adjuvant, (B) turpentine oil and (C) dexamethasone. In the three treatments the more interactive glycoforms increased as compared with the control (see Fig. 5C), and strikingly in rats injected with dexamethasone and turpentine oil.

The information now available allows some generalizations of clinical interest. The Con A-CAIE pattern of AGP [119] and other serum glycoproteins from normal subjects is fairly constant. The Con A interactive or Con A highly interactive glycoforms of AGP, described according to the RC parameter, increase in patients with surgical trauma [69,75], bacterial septicaemia and infection [69,76,77], burns [65,74,78], acute rheumatoid arthritis [81], rheumatoid arthritis with intercurrent infections [78], acute pancreatitis [69] and also in systemic lupus erythematosus with intercurrent infection [120]. All these diseases are characterized by an acute response. In patients with HIV infection, but without open AIDS symptoms, both the concentrations of AGP, PI and ceruloplasmin and the RC for AGP (Con A-CAIE) were normal. However, RC increased at the beginning of AIDS (when infection with *Pneumocystis carinii* was detected). This could be also considered as an acute-phase reaction [121]. On the other hand, the RC of AGP was normal in systemic lupus erythematosus [120] and ischaemic heart desease [79] and lower than normal in subjects with rheumatoid arthritis [77,120] and ankylosing spondylitis [76,122]. Con A-CAIE patterns have also been obtained for other serum glycoproteins in inflammatory processes, but the data are not sufficient to make practical generalizations [9,27,69,84,108,110].

The Fuc content in the glycans of serum proteins increases during inflammation, as de-
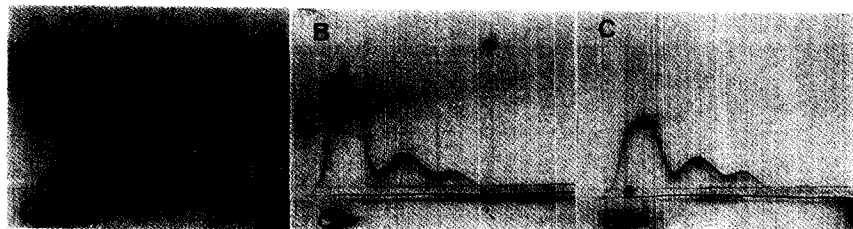

Fig. 7. Con A-CAIE patterns of AGP from sera of adult rat injected with (A) complete Freund's adjuvant, (B) turpentine oil and (C) dexamethasone.

termined by CAIE [123]. For AGP, this increase is mainly due to new sialyl Lewis-X-containing glycans which were detected by CAIE in the presence of AAA. This is an interesting observation, because the above-mentioned structure has been found to be a ligand for some adhesion molecules involved in the traffic of leukocytes to inflammatory areas [9,124]. Thus, the sialyl Lewis-X glycans of AGP could be implicated in the regulation of the intensity and persistence of inflammation [9].

Inflammatory cytokines regulate the hepatic synthesis of the acute-phase proteins [116,117]. As the protein glycosylation also changes during inflammation, several experiments have been carried out, in vivo and in vitro, to make connections between both processes. In human hepatoma cell lines, IL-1, TNF and IL-6 broadly reproduced the changes in glycosylation of PI observed in acute-phase serum [27]. Treatments of human hepatocytes cultures with IL-1 and IL-6 in the presence of dexamethasone resulted in an increase in the Con A strongly reactive AGP microforms [83]. These three cytokines produced a comparable effect on rat AGP in vivo [83,88]. In addition, other factors such as transforming growth factor $\beta 1$, leukaemia inhibitory factor and interferon γ, might also be implicated in the glycosylation of AGP and other serum proteins [84,88,93,125]. In rat AGP from serum of transgenic mice, carrying highly expressed rat AGP genes, the Con A strongly reactive forms predominate. In contrast, in rat AGP produced by cultured hepatocytes isolated from the transgenic mice, a shift towards Con A moderately reactive forms was observed [87]. IL-1 and IL-6 restore the Con A pattern of rat AGP observed in vivo [87]. In conclusion, cytokines involved in inflammation affect the glycosylation pattern of acute-phase and other serum glycoproteins. The effects of these cytokines on the synthesis of acute-phase proteins and on the glycosylation of serum proteins seem to be different. Probably the cytokines affect the expression or the activity of glycosylation enzymes. These enzymes may glycosylate the newly synthesized proteins during their transit in the lumen of endoplasmic reticulum and in the Golgi network, independent of the synthesis rate of each particular protein [27,84,86].

4.2. Cancer

AIE has been applied to study the glycan heterogeneity of AGP and AFP, and also other serum proteins from cancer patients [31,82]. The results obtained for AGP are, however, of limited clinical interest. For most cancer patients the synthesis and glycosylation of serum proteins produced by the liver are independent of the tumoural activity. For example, only a moderate increase in the RC for AGP has been observed, using Con A-CAIE, in individuals suffering from yolk sac tumours [78]. In other types of tumours, RC for AGP was either unaffected [69,78] or even decreased [78,82], as compared with the RC for AGP in healthy individuals.

The serum levels of AFP may increase in patients with hepatocellular carcinoma and yolk sac tumours (reviewed in Ref. [31]). Therefore, AFP should be a reliable tumour marker for the diagnosis of these harmful diseases. However, moderately increased AFP has also been detected in serum of patients with diverse hepatic pathologies and other types of cancers [31]. The glycan analysis of AFP, using CAIE and LAE, was a useful tool to differentiate among these diseases. In general, tumoural cells and their analogous foetal tissues produce similar glycan microforms of AFP. Thus, the Con A reactive fraction predominates either in AFP from patients with hepatocellular carcinomas or in the AFP synthesized by the foetal liver. In contrast, the Con A non-reactive microform greatly increases in AFP from yolk sac tumours (see Fig. 3) and in AFP synthesized by the yolk sac. An intermediate Con A glycosylation pattern appears in the AFP produced by other tumours. Comparable results have been obtained in the analysis of the protein with LCA. In yolk sac tumours, the LCA reactive fractions, and particularly the weakly reactive fraction, increase. The LCA patterns of AFP from hepatocellular carcinoma expressed both the reactive and the

non-reactive fractions. In AFP from patients with hepatitis and cirrhosis, the Con A pattern is comparable to that of AFP from hepatocellular carcinoma. Further discrimination should be obtained using E-PHA and other lectins. For example, the fraction AFP-P4, according to the nomenclature used by Taketa [31], increases in hepatocellular carcinoma. Detailed information about the clinical applications of AFP analysis using AIE techniques can be found elsewhere [31,34,36,39,126–128].

4.3. Pregnancy and foetal development

Several serum proteins have been studied by AIE in relation to pregnancy and foetal development [46,69–71,129,130]. It is well known that AIE analysis of AFP from amniotic fluid, using Con A and other lectins, can detect with almost total confidence defects in the neural tube development (reviewed in Ref. [46]). In these cases the Con A non-reactive fraction of AFP decreased [131]. The LCA analysis of AFP produced similar results to those using Con A [46]. In human pregnancy a complete change in the Con A-CAIE pattern of AGP and other serum proteins was observed [69,104,130]. The principal trend observed was a large increase in the Con A non-reactive fraction. The Con A-CAIE patterns for AGP were similar in maternal sera
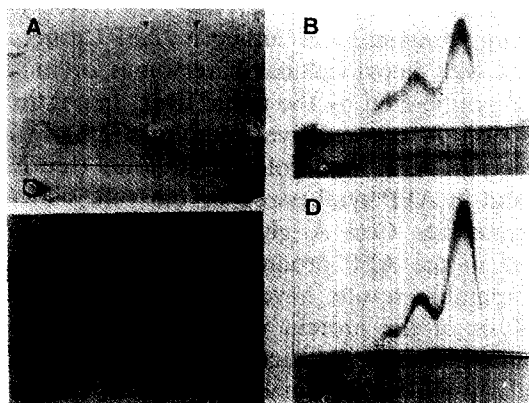

Fig. 8. Con A-CAIE patterns of (A) and (C) fetuin from foetal and adult pig serum, respectively, and (B) and (D) AGP from foetal pig serum at middle and term gestational age.

and amniotic fluid, but different in foetal serum in which the Con A reactive fraction predominates [129]. The Con A non-reactive fraction also increases in the first part of the menstrual cycle and in women receiving estrogen–progestogen treatment [132]. In other species, as illustrated in Fig. 8, significant differences between the Con A pattern of AGP and other proteins, during foetal development, have been observed [70–72].

5. Conclusions

AIE with lectins can be applied almost without restriction to the glycosylation analysis of proteins in biological fluids. Methods of increased sensitivity to detect proteins combined with powerful affinity electrophoretic techniques will extend this type of analysis to glycoproteins present in the serum at very low concentration. Up to now, the major clinical application of AIE in the field of serum proteins has concerned AGP and AFP. The results obtained with these proteins have been useful in differential diagnosis and management of inflammatory processes and in the study of foetal development anomalies.

The number of lectins widely applied in AIE is small. However, the growing information about the properties of other lectins will provide new insights into subtle differences in the glycan structures of proteins. This is particularly exciting in the field of AIE analysis because, in spite of the wide biochemical knowledge of glycan structures, the function of the glycan moieties is still ill-defined. The changes in the glycosylation of many glycoproteins will be implicated in the regulation of inflammation, cellular communication and in general immune responses.

Abbreviations

AAA = *Aleuria aurantia* agglutinin
AFP = α-fetoprotein
AGP = α_1-acid glycoprotein
AIE = affinoimmunoelectrophoresis

allo A = *Allomyrina dichotoma* lectin
CAIE = crossed affinoimmunoelectrophoresis
Con A = concanavalin A
ConA-CAIE = CAIE with Con A
DSA = *Datura stramonium* agglutinin
E-PHA = *Phaseolus vulgaris* isolectin E-4
Fuc = fucose
Gal = galactose
GlcNAc = N-acetylglucosamine
IL-1 = interleukin-1
IL-6 = interleukin-6
LAE = lectin affinity electrophoresis
LCA = *Lens culinaris* agglutinin or lentin lectin
NeuAc = sialic acid
N-glycans = complex-type glycans located in N-
   (Asparagine) glycoprotein sites
O-glycans = complex-type glycans located in O-
   (serine/threonine) glycoprotein sites
PI = α_1-antitrypsin
PSA = *Pisum sativum* agglutinin
RC = reactivity coefficient
TNF = tumour necrosis factor
Tf = transferrin
WGA = wheat germ agglutinin

Acknowledgements

References

[1] N.H.H. Heegaard and T.C. Bøg-Hansen, *Appl. Theor. Electrophoresis*, 1 (1990) 249.
[2] K. Takeo, *Electrophoresis*, 5 (1984) 187.
[3] K. Takeo, in A. Chrambach, M.J. Dunn and B.J. Radola (Editors), *Advances in Electrophoresis*, VCH, Weinheim, 1987, p. 229.
[4] K. Taketa, *J. Chromatogr.*, 569 (1991) 229.
[5] P.M.H. Heegaard, N.H.H. Heegaard and T.C. Bøg-Hansen, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 1, p. 3.
[6] J.U. Baenziger, in F.W. Putnam (Editor), *The Plasma Proteins, Structure, Function and Genetic Control*, Vol. IV, Academic Press, New York, 1984, p. 272.
[7] M.W.C. Hatton, L. März and E. Regoeczi, *Trends Biochem. Sci.*, 8 (1983) 287.
[8] T.W. Rademacher, R.B. Parekh and R.A. Dwek, *Annu. Rev. Biochem.*, 57 (1988) 785.
[9] T.W. De Graaf, M.E. Van der Stelt, M.G. Anbergen and W. van Dijk, *J. Exp. Med.*, 177 (1993) 657.
[10] B. Bayard, J.P. Kerckaert, G. Strecker, L. Dorland, H.V. Halbeek and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 137 (1983) 319.
[11] T. Krusius and E. Ruoslahti, *J. Biol. Chem.*, 257 (1982) 3453.
[12] H. Yoshima, A. Matsumoto, T. Mizuochi, T. Kawasaki and A. Kobata, *J. Biol. Chem.*, 256 (1981) 8476.
[13] E.F. Hounsell, in E.F. Hounsell (Editor), *Glycoprotein Analysis in Biomedicine (Methods in Molecular Biology*, Vol. 14), Humana Press, Totowa, NJ, 1993, Ch. 1, p. 1.
[14] H. Debray and J. Montreuil, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 2, p. 23.
[15] T.C. Bøg-Hansen, *Anal. Biochem.*, 56 (1973) 480.
[16] T.C. Bøg-Hansen, O.J. Bjerrum and J. Ramlau, *Scand. J. Immunol.*, 4, Suppl. 2 (1975) 141.
[17] K. Taketa, E. Ichikawa, H. Taga and H. Hirai, *Electrophoresis*, 6 (1985) 492.
[18] H. Lis and N. Sharon, *Annu. Rev. Biochem.*, 55 (1986) 35.
[19] A.M. Wu, S. Sugii and A. Herp, in T.C. Bøg-Hansen and D.L.J. Freed (Editors), *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 6, Sigma, St. Louis, MO, 1988, p. 723.
[20] P.M.H. Heegaard and T.C. Bøg-Hansen, *Appl. Theor. Electrophoresis*, 3 (1993) 213.
[21] J.P. Salier, L. Faye, D. Vergaine and J.P. Martin, *Electrophoresis*, 1 (1980) 193.
[22] L. Faye and J.P. Salier, *Electrophoresis*, 10 (1989) 841.
[23] T.J. Hahn and C.F. Goochee, *Anal. Biochem.*, 199 (1991) 243.
[24] N.H.H. Heegaard, M. Hagerup, Å.C. Thomsen and P.M.H. Heegaard, *Electrophoresis*, 10 (1989) 836.
[25] B. Mallet, J.L. Franc and M.C. Zattara, *FEBS Lett.*, 262 (1990) 36.
[26] O. Pos, A. Drechou, G. Durand, M.F.A. Bierhuizen, M.E. Van der Stelt and W. Van Dijk, *Clin. Chim. Acta*, 184 (1989) 121.
[27] A. Mackiewicz, M.K. Ganapathi, D. Schultz and I. Kushner, *J. Exp. Med.*, 166 (1987) 253.
[28] P.C. Kelleher, K. Toftager-Larsen and C.J.P. Walters, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 6, p. 89.
[29] W. van Dijk and M.E. van der Stelt, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 5, p. 81.

[30] K. Taketa, E. Ichikawa, K. Umetsu and T. Suzuki, *Cancer Lett.*, 31 (1986) 325.

[31] K. Taketa, *Hepatology*, 12 (1990) 1420.

[32] J.P. Kerckaert, B. Bayard, F. Puech, X. Codaccioni and G. Biserte, *Clin. Chim. Acta*, 104 (1980) 245.

[33] K. Toftager-Larsen, in J. Hau (Editor), *Pregnancy Proteins in Animals*, Walter de Gruyter, Berlin, 1986, p. 41.

[34] Y. Aoyagi, Y. Suzuki, M. Isemura, K. Soga, T. Ozaki, T. Ichida, K. Inoue, H. Sasaki and F. Ichida, *Gann*, 75 (1984) 809.

[35] T. Ishiguro, H. Sakaguchi, M. Fukui and I. Sugitachi, *Tumour Biol.*, 6 (1985) 195.

[36] K. Taketa and H. Hirai, *Electrophoresis*, 10 (1989) 562.

[37] K. Shimizu, T. Taniichi, S. Satomura, S. Matsuura, H. Taga and K. Taketa, *Clin. Chim. Acta*, 214 (1993) 3.

[38] H. Hirai and K. Taketa, *J. Chromatogr.*, 604 (1992) 91.

[39] K. Taketa, Y. Endo, C. Sekiya, K. Tanikawa, T. Koji, H. Taga, S. Satomura, S. Matsuura, T. Kawai and H. Hirai, *Cancer Res.*, 53 (1993) 5419.

[40] K. Taketa, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 14, p. 199.

[41] K. Ohkawa, K. Takada, N. Takizawa, T. Hatano, Y. Tsukada and M. Matsuda, *FEBS Lett.*, 270 (1990) 19.

[42] K. Taketa, E. Ichikawa, J. Sato, H. Taga and H. Hirai, *Electrophoresis*, 10 (1989) 825.

[43] K. Taketa, Y. Fujii, T. Aoi, H. Taga and S. Nishi, *Electrophoresis*, 14 (1993) 798.

[44] D. Gitlin, *Ann. N.Y. Acad. Sci.*, 259 (1975) 7.

[45] H.F. Deutsch, *Adv. Cancer Res.*, 56 (1991) 253.

[46] K. Toftager-Larsen, *Dan. Med. Bull.*, 37 (1990) 41.

[47] Y. Tsuchida, K. Yamashita, A. Kobata, S. Nishi, Y. Endo and S. Saito, *Tumour Biol.*, 5 (1984) 33.

[48] D.C. Parmelee, M.A. Evenson and H.F. Deutsch, *J. Biol. Chem.*, 253 (1978) 2114.

[49] F. Lampreave, M. Calvo, J. Naval and A. Piñeiro, *Comp. Biochem. Physiol.*, 73B (1982) 823.

[50] M. Calvo, J. Naval, F. Lampreave and A. Piñeiro, *Biochim. Biophys. Acta*, 959 (1988) 238.

[51] K. Yamashita, K. Taketa, S. Nishi, K. Fukushima and T. Ohkura, *Cancer Res.*, 53 (1993) 2970.

[52] H. Yoshima, T. Mizuochi, M. Ishii and A. Kobata, *Cancer Res.*, 40 (1980) 4276.

[53] J.P. Kerckaert, B. Bayard and G. Biserte, *Biochim. Biophys. Acta*, 576 (1979) 99.

[54] J.P. Kerckaert and B. Bayard, in T.C. Bøg-Hansen (Editor), *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 1, Walter de Gruyter, Berlin, 1981, p. 271.

[55] A. Mackiewicz and J. Breborowicz, *Oncodev. Biol. Med.*, 1 (1980) 251.

[56] Y. Aoyagi, M. Isemura, Z. Yosizawa, Y. Suzuki, C. Sekine, T. Ono and F. Ichida, *Biochim. Biophys. Acta*, 830 (1985) 217.

[57] J. Breborowicz and K. Gryska, in T.C. Bøg-Hansen and D.L.J. Freed (Editors), *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 6, Sigma, St. Louis, MO, 1988, p. 497.

[58] K. Taketa, Y. Fujii and H. Taga, *Electrophoresis*, 14 (1993) 1333.

[59] K. Schmid, in P. Baumann, C.B. Eap, W.E. Müller and J.P. Tillement (Editors), *Alpha₁-Acid Glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology*, Alan R. Liss, New York, 1989, p. 7.

[60] I. Kushner, *Methods Enzymol.*, 163 (1988) 373.

[61] K. Schmid, J. Emura, M.F. Schmid, R.L. Stevens and R.B. Nimberg, *Int. J. Pept. Protein Res.*, 11 (1978) 42.

[62] H. Toh, H. Hayashida, R. Kikuno, T. Yasunaga and T. Miyata, *Nature*, 314 (1985) 191.

[63] F. Lampreave, N. González-Ramón, S. Martínez-Ayensa, M.A. Hernández, H.K. Lorenzo, A. García-Gil and A. Piñeiro, *Electrophoresis*, 15 (1994) 672.

[64] M. Bennett and K. Schmid, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6109.

[65] W. van Dijk, O. Pos, M.E. van der Stelt, H.J. Moshage, S.H. Yap, L. Dente, P. Baumann and C.B. Eap, *Biochem. J.*, 276 (1991) 343.

[66] M.F.A. Bierhuizen, M. De Wit, C.A.R.L. Govers, W. Ferwerda, C. Koeleman, O. Pos and W. Van Dijk, *Eur. J. Biochem.*, 175 (1988) 387.

[67] O. Pos, W. Van Dijk, N. Ladiges, C. Linthorst, M. Sala, D. Van Tiel and W. Boers, *Eur. J. Cell. Biol.*, 46 (1988) 121.

[68] I. Nicollet, J.P. Lebreton, M. Fontaine and M. Hiron, *Biochim. Biophys. Acta*, 668 (1981) 235.

[69] J. Raynes, *Biomedicine*, 36 (1982) 77.

[70] F. Lampreave and A. Piñeiro, *Int. J. Biochem.*, 16 (1984) 47.

[71] F. Lampreave, M.A. Alava and A. Piñeiro, *Electrophoresis*, 14 (1993) 214.

[72] H. Itoh, K. Tamura, M. Izumi, Y. Motoi and Y. Funayama, *Am. J. Vet. Res.*, 54 (1993) 591.

[73] W. van Dijk, O. Pos, M.E. Van der Stelt and M.F.A. Bierhuizen, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 7, p. 97.

[74] T. Pawlowski, M. Biczysko, M. Solarewicz and S. Mackiewicz, in T.C. Bøg-Hansen and D.L.J. Freed (Editors), *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 6, Sigma, St. Louis, MO, 1988, p. 491.

[75] J.E.S. Hansen, S.P. Jensen, B. Nørgaard-Pedersen and T.C. Bøg-Hansen, *Electrophoresis*, 7 (1986) 180.

[76] K. Fassbender, W. Zimmerli, R. Kissling, M. Sobieska, A. Aeschlimann, M. Kellner and W. Müller, *Clin. Chim. Acta*, 203 (1991) 315.

[77] K. Fassbender, H. Michels, F. Zepp, W. Zimmerli, A. Aeschlimann, S. Mackiewicz and W. Müller, *J. Rheumatol.*, 20 (1993) 123.

[78] J. Breborowicz and A. Mackiewicz, *Electrophoresis*, 10 (1989) 568.

[79] L.E. Pedersen, J. Bonde, N.A. Graudal, N.V. Backer, J.E.S. Hansen and J.P. Kampmann, *Br. J. Clin. Pharmacol.*, 23 (1987) 41.

[80] A. Mackiewicz, T. Pawlowski, A. Mackiewicz-Pawlowska, K. Wiktorowicz and S. Mackiewicz, *Clin. Chim. Acta*, 163 (1987) 185.

[81] P. Hrycaj, M. Sobieska, S. Mackiewicz and W. Muller, *Ann. Rheum. Dis.*, 52 (1993) 138.

[82] J. Breborowicz, A. Gorny, K. Drews and A. Mackiewicz, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 13, p. 191.

[83] O. Pos, H.J. Moshage, S.H. Yap, J.P. Snieders, L.A. Aarden, J. Van Gool, W. Boers, A.M. Brugman and W. Van Dijk, *Inflammation*, 13 (1989) 415.

[84] A. Mackiewicz and I. Kushner, *Electrophoresis*, 10 (1989) 830.

[85] A. Mackiewicz, O. Pos, M. van der Stelt, S.H. Yap, M. Kapcinska, M. Laciak, M.J. Dewey, F.G. Berger, H. Baumann, I. Kuhsner and W. van Dijk, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 9, p. 135.

[86] M. Hiron, M. Daveau and J.P. Lebreton, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 11, p. 163.

[87] A. Mackiewicz, M.J. Dewey, F.G. Berger and H. Baumann, *Glycobiology*, 1 (1991) 265.

[88] C. Poüs, L. Chauvetot-Moachon, M. Lecoustillier and G. Durand, *Inflammation*, 16 (1992) 197.

[89] J. Travis and G.S. Salvensen, *Annu. Rev. Biochem.*, 52 (1983) 655.

[90] T. Mega, E. Lujan and A. Yoshida, *J. Biol. Chem.*, 255 (1980) 4057.

[91] L.C. Hodges, R. Laine and S.K. Chan, *J. Biol. Chem.*, 254 (1979) 8208.

[92] B. Bayard, J.P. Kerckaert, A. Laine and A. Hayem, *Eur. J. Biochem.*, 124 (1982) 371.

[93] A. Mackiewicz, M. Laciak, A. Górny and H. Baumann, *Eur. J. Cell Biol.*, 60 (1993) 331.

[94] S. Duthel and A. Revol, *Clin. Chim. Acta*, 215 (1993) 173.

[95] D. Thisner and Å. Rosengren, *Electrophoresis*, 3 (1982) 226.

[96] P. Arnaud, L. Miribel and D.L. Emerson, *Methods Enzymol.*, 163 (1988) 431.

[97] D.L. Christie, K.M. Dziegielewska, R.M. Hill and N.R. Saunders, *FEBS Lett.*, 214 (1987) 45.

[98] F. Lampreave and A. Piñeiro, *J. Reprod. Fertil.*, 95 (1992) 441.

[99] E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson and H. Van Halbeek, *J. Biol. Chem.*, 263 (1988) 18253.

[100] G. Rauth, O. Pöschke, E. Fink, M. Eulitz, S. Tippmer, M. Kellerer, H.U. Häring, P. Nawratil, M. Haasemann, W. Jahnen-Dechent and W. Müller-Esterl, *Eur. J. Biochem.*, 204 (1992) 523.

[101] M. Jezequel, N.S. Seta, M.M. Corbic, J.M. Feger and G.M. Durand, *Clin. Chim. Acta*, 176 (1988) 49.

[102] R.T.A. MacGillivray, E. Mendez, G.S. Jaiprakash, S.K. Sinha, J. Lineback-Zins and K. Brew, *J. Biol. Chem.*, 258 (1983) 3543.

[103] G. Spik, V. Debruyne, J. Montreuil, H. van Halbeek and J.F.G. Vliegenthart, *FEBS Lett.*, 183 (1985) 65.

[104] D. Léger, B. Campion, J.P. Decottignies, J. Montreuil and G. Spik, *Biochem. J.*, 257 (1989) 231.

[105] J.H. Brock, in P.M. Harrison (Editor), *Metalloproteins, Part 2*, Macmillan, London, 1985, p. 183.

[106] K. Ohkawa, K. Takada, N. Takizawa, T. Hatano, Y. Tsukada and M. Matsuda, *FEBS Lett.*, 270 (1990) 19.

[107] B. Campion, D. Léger, J.M. Wieruszeski, J. Montreuil and G. Spik, *Eur. J. Biochem.*, 184 (1989) 184, 405.

[108] A. Laine and E. Hachulla, *Ann. Biol. Clin.*, 49 (1991) 359.

[109] A. Laine, E. Hachulla and A. Hayem, *Electrophoresis*, 10 (1989) 227.

[110] W. Dobryszycka and I. Katnik, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 15, p. 211.

[111] K. Okajima, H. Ueyama, Y. Hashimoto, Y. Sasaki, K. Matsumoto, H. Okabe, M. Inoue, S. Araki and K. Takatsuki, *Thromb. Haemostas.*, 61 (1989) 20.

[112] M.J. Sinosich, *Electrophoresis*, 11 (1990) 70.

[113] M. Belle, M. Hanss, M. Guillaumont, M. Leclercq and R. Guinet, *Electrophoresis*, 12 (1991) 294.

[114] T. Hada and K. Higashino, *Physico.-Chem. Biol.*, 34 (1990) 247.

[115] S.B. Rosalki and A.Y. Foo, *Electrophoresis*, 10 (1989) 604.

[116] H. Baumann and J. Gauldie, *Mol. Biol. Med.*, 7 (1990) 147.

[117] P.C. Heinrich, J.V. Castell and T. Andus, *Biochem. J.*, 265 (1990) 621.

[118] O. Poss, A. Drechou, G. Durand, M.F.A. Bierhuizen, M.E. van der Stelt and W. van Dijk, in P. Baumann, C.B. Eap, W.E. Müller and J.P. Tillement (Editors), *Alpha₁-Acid Glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology*, Alan R. Liss, New York, 1989, p. 279.

[119] J.E.S. Hansen, T.C. Bøg-Hansen, B. Pedersen and K. Neland, *Electrophoresis*, 10 (1989) 574.

[120] A. Mackiewicz, R. Marcinkowska-Pieta, S. Ballou, S. Mackiewicz and I. Kushner, *Arthritis Rheum.*, 30 (1987) 513.

[121] J.E.S. Hansen and C. Pedersen, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 18, p. 251.

[122] A. Mackiewicz, M.A. Khan, T.L. Reynolds, S. Van der Linden and I. Kushner, *Ann. Rheum. Dis.*, 48 (1989) 99.

[123] D. Biou, D. Konan, J. Féger, J. Agneray, Y. Leroy, P. Cardon, B. Fournet and G. Durand, *Biochim. Biophys. Acta*, 913 (1987) 308.

[124] T.A. Springer and L.A. Lasky, *Nature*, 349 (1991) 196.

[125] A. Mackiewicz and I. Kushner, *Inflammation*, 14 (1990) 485.

[126] J. Breborowicz, D. Breborowicz and A. Mackiewicz, in J. Breborowicz and A. Mackiewicz (Editors), *Affinity Electrophoresis: Principles and Application*, CRC Press, Boca Raton, FL, 1992, Ch. 20, 265.

[127] T. Ishiguro and Y. Takahashi, *Dis. Markers*, 7 (1989) 239.

[128] K. Taketa, M. Liu, C. Sekiya, H. Kanagawa, A. Ohmori, M. Sato and H. Taga, *J. Tumor Marker Oncol.*, 9 (1994) 69.

[129] N. Seta, B. Tissot, F. Forestier, J. Feger, F. Daffos and G. Durand, *Clin. Chim. Acta*, 203 (1991) 167.

[130] D. Biou, C. Bauvy, H. N'Guyen, P. Codogno, G. Durand and M. Aubery, *Clin. Chim. Acta*, 204 (1991) 1.

[131] K. Toftager-Larsen and B. Nørgaard-Pedersen, *Clin. Genet.*, 33 (1988) 220.

[132] M. Succari, M.J. Foglietti and F. Percheron, *Clin. Chim. Acta*, 187 (1990) 235.