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Determination of sulfate ester content in sulfated oligo- and poly-saccharides by capillary electrophoresis with indirect UV detection

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ABSTRACT: Carbohydrates having sulfate groups such as glycosaminoglycans and chemically synthesized sucrose sulfate show interesting and important biological activities. We adapted CE with indirect UV detection technique to the determination of sulfate ester in sulfated carbohydrates, which were previously hydrolyzed with HCl. The liberated sulfate ion was analyzed using a background electrolyte consisting of triethanolamine-buffered chromate with hexamethonium bromide. Sulfate contents of glucose 3-sulfate and sucrose octasulfate used as a model were in good agreement with theoretical values (accuracy, 95.9–96.7 and 97.4–101.9%, respectively), and relative standard deviation values run-to-run were 0.977 and 1.90%, respectively. We applied the method to the determination of the sulfate contents of some glycosaminoglycan samples and showed that the contents were in good agreement with those calculated from sulfur content. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: sulfate; glycosaminoglycans; capillary electrophoresis indirect UV detection

Introduction

Sulfation of hydroxyl and amino groups (-O-SO_3H and -NH-SO_3H) is one of the common modifications of carbohydrates and is often observed in various glycoconjugates such as proteoglycans or mucin-like glycoproteins. Sulfated carbohydrates are widely distributed in animals as the major constituents of proteoglycans and are biologically active molecules involved in various biological events (Hooper *et al.*, 1996; Honke and Taniguchi, 2002; Wu, 2006). Functions of sulfated carbohydrates strongly depend on the presence and spatial positioning in the molecules. Degree of sulfation on carbohydrates is also closely related to biological activities such as blood coagulation, signal transduction and cell–cell interaction (Lindahl *et al.*, 1983; Villanueva, 1984; Hemmerich and Rosen, 1994; Small *et al.*, 1996; Tsuboi *et al.*, 1996). Chemical sulfation of carbohydrates often affords compounds showing novel biological activities such as anti-HIV activities (Katsuraya *et al.*, 1994, 1999; Yoshida *et al.*, 1995; Hattori *et al.*, 1998). Sulfated carbohydrates also have potential as pharmaceuticals (Werz and Seeberger, 2005). Sucrose octasulfate, 'Sucralfate' and the chemically synthesized octasulfated pentasaccharide 'Arixtra' are used as antiulcer and anticoagulant drugs, respectively (Candelli *et al.*, 2000; Giangrande, 2002).

In view of these interesting features of sulfated carbohydrates, assessment of sulfate content is important not only for the understanding of their biological significance but also the development and manufacturing of novel bioactive sulfated carbohydrates. Several methods have been developed for the determination of sulfate content of carbohydrates. Classical methods are based on the colorimetric determination of the inorganic sulfate ion liberated from sulfated carbohydrates by acid hydrolysis, such as chelating barium ions with rhodizonate (Terho and Hartiala, 1971; Roy and Turner, 1982). Srinivasan *et al.* achieved the determination of microgram quantity of sulfate ion based on the formation of stable complex of sulfate ester with *n*-butylamine and achieved determination of microgram quantity of sulfate ion (Srinivasan *et al.*, 1970). Unfortunately, these methods are not suitable for the determination of a small amount of sulfate ester in complex carbohydrate, because they are time-consuming and not sensitive enough, require a significant amount of material, and are prone to interference from the other ions. Compared with these conventional methods, ion chromatography (IC) demonstrates increased specificity and sensitivity

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Abbreviations used: BGE, background electrolyte; CSA, chondroitin sulfate A; DMF, *N,N*-dimethylformamide; DS, dermatan sulfate; GAG, glycosaminoglycan; HA, hyaluronic acid; HMB, hexamethonium bromide; HP, heparin; HS, heparan sulfate; TBA, tributylamine; TEA, triethanolamine.

as well as the inherent ability for the determination of various inorganic ions, and has been applied to the analysis of the ions in the samples from biological, environmental and industrial origins (Lopez-Ruiz, 2000). The sulfate contents in glycoproteins or GAGs were successfully determined by IC (Cole and Evrovski, 1997; Toida *et al.*, 1999). Toida *et al.* liberated the sulfate ion from chemically O-sulfated GAGs by acid hydrolysis, and determined it by a combination of IC and conductivity detection (Tadano-Aritomi *et al.*, 2001).

Capillary electrophoresis (CE) is a powerful tool for separation of inorganic ions with high resolving power. Its performance is comparable with that of IC, and has become one of the standard tools for the analysis of inorganic ions in environmental, biomedical, clinical and industrial samples (Fritz, 2000; Timerbaev, 2002, 2004; Johns *et al.*, 2003; Pacakova *et al.*, 2003; Paull and King, 2003). CE allows rapid analysis with high resolution and exhibits good capabilities in quantitative analysis, making it well suited for routine analysis of sulfate content of carbohydrates. Although the detection in CE is usually performed by direct UV detection, most inorganic ions lack a chromophore and cannot be detected using common direct UV detection. Therefore, indirect UV detection technique is usually used for determination of inorganic ions. Indirect UV detection adds an UV-absorbing co-ion (called the probe) to the background electrolyte (BGE) and this probe is displaced by migration, causing a negative signal. Indirect UV detection is an effective alternative detection technique for inorganic ions. The attractive performance of the CE method has been employed for the assay of sulfotransferase activity (Saillard *et al.*, 1999). Thus, CE is considered a useful alternative to the well-established IC method for routine analysis of sulfate content of carbohydrates.

In the present study, we developed a method using capillary electrophoresis with indirect UV detection to the determination of sulfate content of sulfated oligo-/polysaccharides, and applied the method for the determination of sulfate content in some sulfated GAGs and the monitoring of chemically sulfation reaction of polysaccharides. The present method will provide a robust method for the analysis of sulfated carbohydrates using routinely available laboratory instrumentation.

Experimental

Materials

Hexamethonium bromide, glucose 3-sulfate (sodium salt, 98% purity by HPLC), sucrose octasulfate (sodium salt) and heparin from bovine intestinal mucosa were purchased from Sigma (St Louis, MO, USA). Hyaluronic acid (from *Streptococcus zooepidemicus*) was purchased from Nacalai tesque (Uji, Kyoto, Japan). Chondroitin sulfate A (from whale cartilage), dermatan sulfate (from pig skin) and heparan sulfate (from bovine kidney) were purchased from Seikagaku Biobusiness (Chiyoda-ku, Tokyo, Japan). Tributylamine (TBA), *N,N*-dimethylformamide (DMF) and pyridine–sulfur trioxide complex were obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). All other chemicals and reagents were of the highest grade or HPLC grade. Running buffers and aqueous solutions were prepared with water purified with a Milli-Q purification system (Millipore, Bedford, MS, USA).

Sulfation of Chondroitin Sulfate A

Chemical sulfation of chondroitin sulfate A was performed according to the method reported by Maruyama *et al.* (1998). The sodium salt (10 mg) of chondroitin sulfate A (from whale cartilage) was dissolved in 1 mL of 5% TBA–HCl water (pH 2.8), and then the solution was lyophilized to dryness to give the tributylammonium salt. The salt was dissolved in 1 mL of DMF, and pyridine–sulfur trioxide complex (10, 50, 100 and 250 mg) was added. After incubating the mixture for 1 h at 40°C, the reaction was terminated by addition of water (1 mL). The reaction product was precipitated with cold ethanol (6 mL) saturated with anhydrous sodium acetate, collected by centrifugation at 4°C, then dissolved in water followed by dialysis against water to remove salts and lyophilized. We obtained $PSCS_{10}$ (11.8 mg), $PSCS_{50}$ (14.1 mg), $PSCS_{100}$ (17.2 mg) and $PSCS_{250}$ (18.6 mg), respectively, by changing the amount of pyridine–sulfur trioxide complex.

Sample and Standard Solutions

Standard solutions of sulfate ion were prepared by dissolving an accurately weighed amount of sodium sulfate (300 mg) in water (10 mL; 210 mM). A series of standard solutions of sulfate ion for calibration curve were prepared by appropriate dilution of the standard solution with water. Sample solution of sulfated oligo-/polysaccharides was also prepared by dissolving an accurately weighed amount (1.00 mg) in of water (1 mL).

CE Analysis of Sulfate Ion with Indirect UV Detection

CE was performed with a Beckman P/ACE MDQ system equipped with a UV detector (Beckman Coulter, Fullerton, CA, USA). A fused silica capillary (50 μm i.d., 56 cm effective length, 66 cm total length, from Agilent Technologies) was used throughout the work. The background electrolyte was composed of 10 mM CrO_3–2 mM hexamethonium bromide in 10% MeOH–water (pH 8.0) adjusted with triethanolamine. The background electrolyte was passed through a cellulose acetate membrane filter (0.2 μm). Prior to the first run, the capillary was rinsed with 0.1 M NaOH for 10 min, followed by washing with water for 10 min, and then filled with the background electrolyte. The capillary was conditioned by pre-electrophoresis (–20 kV) for 10 min. After washing the capillary with water and filling with the background electrolyte, samples were automatically injected using pressure injection mode at 1.0 psi for 10 s. Electrophoresis was performed at –20 kV using reverse polarity. Detection was carried out with monitoring the UV absorption at 254 nm. The negative peaks due to the presence of anions in the background of CrO_3 were automatically converted into positive peaks by Beckman 32 Karat software version 4.0 (Beckman Coulter).

Hydrolysis of Sulfated Carbohydrates

A standard solution (20 μL) of glucose 3-sulfate was mixed with 20 μL of 1 M HCl and the mixture was kept at 100°C in a polypropylene tube for specified times. After cooling the mixture to room temperature, the solution (40 μL) was diluted with 1000 μL of water. An aqueous solutions (1000 μL) of $NaNO_3$ was added to the mixture as internal standard and an aliquot (20 μL) was used for the analysis of sulfate ion.

Standard Procedure for the Determination of Sulfate Content in Sulfated Oligo- and Polysaccharides

A solution (20 μL) of sodium salt of sulfated oligo- or polysaccharides was mixed with 20 μL of 1 M HCl, and the mixture was kept at 100°C for 2 h. After cooling the mixture to room temperature, water and the internal standard solutions were added to the mixture as described above and an aliquot was used for the determination of sulfate ion. The content of sulfate ion was calculated from the calibration curve obtained from standard solutions of Na_2SO_4. The percentages of sulfate content were calculated using the following equation:

$$SO_3\,(w/w) = (\text{wt of } SO_4^{2-} \times (\text{wt of } SO_3/\text{wt of } SO_4)/\text{wt of sample}) \times 100$$

where wt (weight) of $SO_3 = 80$, wt of $SO_4^{2-} = 96$, wt of SO_4^{2-} (μg) in hydrolysates is the amount calculated using the calibration curve, and wt of the sample is the amount of the sample in micrograms.

Results and Discussion

Principle

The method is based on the acid hydrolysis of sulfonic acid ester (-O-SO_3H and -NH-SO_3H) followed by the determination of the released sulfate ion (SO_4^{2-}) with CE with indirect UV detection technique. Sulfated oligo-/polysaccharides are hydrolyzed with HCl to produce SO_4^{2-}. In CE, indirect detection is conveniently available for the detection of compounds which do not have chromophores/fluorophores. When non-UV absorbing sulfate ion passes through the UV detector, the zone of SO_4^{2-} causes a negative signal in the background electrolyte containing a UV-absorbing compound as probe. The output polarity of the detection is reversed so that a positive peak is obtained. The content of sulfate ester in parent compounds is calculated using the calibration curve obtained from a standard solution of Na_2SO_4.

Selection of the Background Electrolyte

Selection of the electrolyte (e.g. co-ion and electroosmotic modifier) is important for the sensitive and quantitative determination of sulfate ion. In the present study, chromate ion was selected as the UV absorbing co-ion (probe ion) because of its ionic mobility being close to that of SO_4^{2-}, which ensures high peak symmetry (Johns *et al.*, 2003; Pacakova *et al.*, 2003). We had to pay attention on the presence of high excess amount of chloride ion (Cl^-) in the sample solutions due to the HCl employed for the hydrolysis of sulfate-containing carbohydrates. Inorganic anions by CE are usually analyzed under negative polarity using electroosmotic modifiers such as cationic surfactant, polymer and amines, which improve resolutions of the ions (Haddad *et al.*, 1999; Harakuwe *et al.*, 1999; Kaniansky *et al.*, 1999). Muzikar *et al.* (2003) reported the determination of trace amount of inorganic anions (e.g. SO_4^{2-} or NO_3^-) in the presence of a large excess of Cl^- using an electrolyte consisting of triethanolamine (TEA)-buffered chromate with hexamethonium bromide (HMB) as electroosmotic modifier. In the present study, SO_4^{2-} (0.21 mM as Na_2SO_4) was successfully analyzed in the presence of 50 mM HCl using this condition, and Cl^- and SO_4^{2-} were completely resolved and observed at 4.50 and 4.85 min, respectively (Fig. 1a).

We employed nitrate ion (NO_3^-) as internal standard (50 μg/mL, 0.59 mM; Fig. 1b). Ions of SO_4^{2-} and NO_3^- with a huge amount of Cl^- were completely resolved within 5 min. Based on these results, 10 mM CrO_3–2 mM HMB in 10% MeOH–water (pH 8.0 adjusted with TEA) was selected as the background electrolyte throughout the present study.

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Linearity and Limit of Detection

The calibration curve for absolute peak area of sulfate ion showed good linearity between 5.0 and 625 μg/mL ($y = 65.4x + 0.58$, $R = 0.9996$; Fig. 2a). In the case of correction of the injection amount by internal standard, the calibration curve exhibited excellent linearity ($y = 0.015x + 0.22$, $R = 0.9999$; Fig. 2b). Both lower limit of detection (LOD) and lower limit of quantification (LOQ) were evaluated on the basis of the standard deviation (σ) and slope (S) from the calibration curve of SO_4^{2-}. In the present conditions, LOD (= 3σ/S) and LOQ (= 10σ/S) were 0.934 and 3.113 μg/mL, respectively.

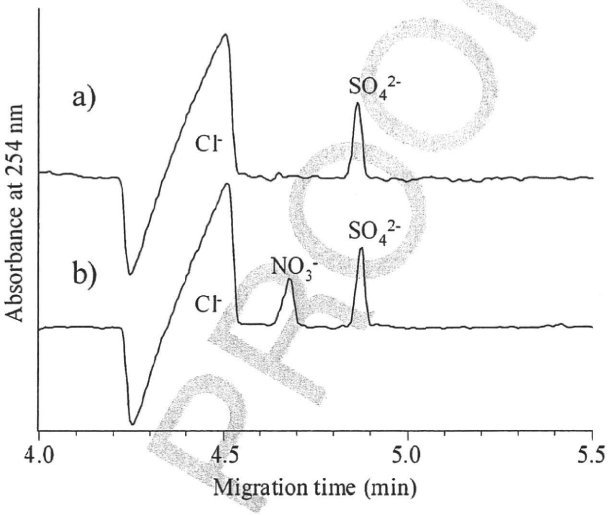

Figure 1. Separation of inorganic anions by CE with indirect UV detection: (a) 0.21 mM sulfate and 50 mM chloride; (b) 0.21 mM sulfate, 50 mM chloride, and 0.59 mM nitrate. Background electrolyte: 10 mM CrO_3/2 mM hexamethonium bromide in 10% MeOH–water at pH 8.0 adjusted with triethanolamine. Capillary: a fused silica capillary (i.d., 50 μm; effective length 56 cm). Applied voltage, –20 kV; temperature, 25°C; sample injection, hydrodynamic injection (1.0 psi, 10 s); detection, indirect UV absorption at 254 nm.

°C

Reproducibility

Run-to-run reproducibility of migration times of SO_4^{2-} and NO_3^- was evaluated using a mixture of 30 μg/mL Na_2SO_4 and 50 μg/mL $NaNO_3$. Migration times of SO_4^{2-} and NO_3^- were 4.96 ± 0.08 and 4.68 ± 0.07, respectively. The relative standard deviation (RSD) was less than 1.4 and 1.6%, respectively ($n = 5$).

Precision

We obtained RSD values in absolute determination of SO_4^{2-} using standard solutions of Na_2SO_4 at 5, 30 and 312.5 μg/mL. The RSD (%) of SO_4^{2-} peak area were 4.58, 2.24 and 2.83%, respectively ($n = 5$; Table 1). In contrast, when using the internal standard (NO_3^-), the RSD (%) of SO_4^{2-} was 1.84, 0.69 and 1.69%, respectively ($n = 5$; Table 1).

Optimization of Conditions for Liberation of SO_4^{2-} by Acid Hydrolysis

Conditions for liberation of SO_4^{2-} from sulfated carbohydrates by acid hydrolysis with HCl were optimized using glucose 3-sulfate as model. After hydrolysis of glucose 3-sulfate with 1 M HCl at 100°C for specified intervals, a portion of the reaction mixture was diluted with water. An aqueous solution of the internal standard ($NaNO_3$) was added, and the released SO_4^{2-} was determined according to the conditions described above.

The content of SO_4^{2-} in the reaction mixture was dependent on hydrolysis time and the hydrolysis was completed within 2 h as shown in Fig. 3(a, b). The excess chloride ion and free glucose produced by hydrolysis in the mixture did not show interference in the determination of SO_4^{2-}. The amount of sulfate ion in glucose 3-sulfate was estimated as 28.1 w/w% (0.96 mol/mol), and showed good agreement with the theoretical values (28.4%). Recoveries were 95.9–96.7% ($n = 5$).

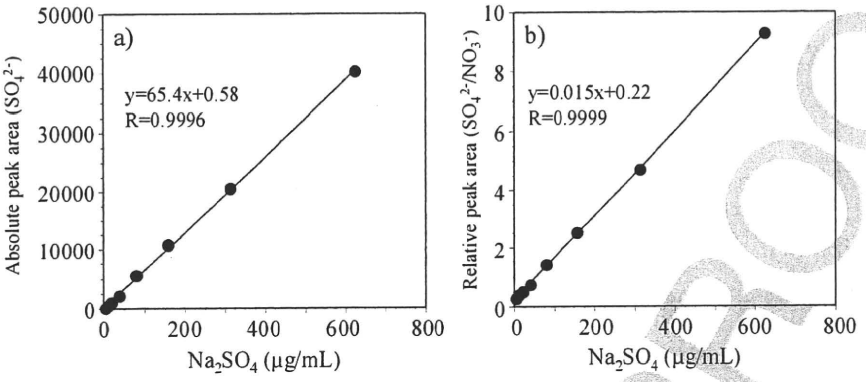

Figure 2. Calibration curve for determination of sulfate ions. (a) Concentration of Na_2SO_4 vs absolute peak area of SO_4^{2-}. (b) Concentration of Na_2SO_4 vs relative peak area (SO_4^{2-}/NO_3^-).

Table 1. Precision results of determination of sulfate ion at three different concentrations

Run	Peak area								
	5 μg/mL Na_2SO_4			30 μg/mL Na_2SO_4			312.5 μg/mL Na_2SO_4		
	SO_4^{2-}	NO_3^-	SO_4^{2-}/NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}/NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}/NO_3^-
1	462	361	1.280	2755	2043	1.349	28041	21505	1.304
2	492	378	1.302	2647	1957	1.353	29945	22869	1.309
3	506	402	1.259	2798	2092	1.337	28762	22214	1.295
4	476	362	1.315	2695	1989	1.355	27945	22290	1.254
5	518	409	1.267	2679	2008	1.334	29013	22452	1.292
Average	491	382	1.284	2715	2018	1.346	28741	22266	1.291
SD	22.5	22.3	0.024	60.8	51.9	0.009	813.6	495.1	0.022
RSD%	4.58	5.83	1.838	2.24	2.57	0.687	2.83	2.22	1.694

We evaluated linearity, repeatability, precision and lower limit of detection using sucrose octasulfate. Sucrose octasulfate is a cytoprotective drug widely used to prevent or treat several gastrointestinal diseases such as gastro-esophageal reflux, gastritis, peptic ulcer, stress ulcer and dyspepsia (Lam and Ching, 1994; Candelli *et al.*, 2000). The sulfate content found in the hydrolysate of sucrose octasulfate showed a good linear relationship with sucrose octasulfate (0.03–1 mg/mL). The value for the relative standard deviation ($n = 5$) of determination of sucrose octasulfate was 1.90% at 250 μg/mL. The limit of detection was 7.8 μg/mL as a solution of sucrose octasulfate sodium salt. When a solution (250 μg/mL) of sucrose octasulfate sodium salt was used, the sulfate content of one batch was 53.1% (accuracy 97.4–101.9%, $n = 5$), which is very close to the theoretical value (54.2%).

Determination of Sulfate Content in Various GAG Samples

Glycosaminoglycans (GAGs) are a family of highly complex and polydisperse linear polysaccharides that display a variety of important biological roles (Jackson *et al.*, 1991; Scott, 1992; Bourin and Lindahl, 1993; Sugahara and Kitagawa, 2000). GAGs are categorized into some main structural groups: hyaluronic acid (HA), chondroitin sulfate A (CSA), dermatan sulfate (DS), heparin (HP) and heparan sulfate (HS) (Zaia, 2009). The structural complexity is compounded by their sequence heterogeneity, primarily caused by variation of the degree and position of sulfate groups. We applied the present method to the determination of sulfate content in some GAG samples. The results are shown in Fig. 4 and Table 2.

Among five GAG samples used in the study, CSA, DS, HP and HS showed sulfate contents of 14.2, 15.0, 25.1 and 11.5%, respectively. HP is mainly composed of trisulfated disaccharide units, α(1-4)-linked L-iduronic acid, which is 2-*O*-sulfated, and D-glucosamine, which is *N*- and 6-*O*-sulfated (Zaia, 2009). Therefore, the sulfate content of HP is higher than those of CSA and DS, which are sulfated at only 4-OH of GalNAc in the disaccharide unit. The sulfate content of HS is lower than those of other sulfated GAGs, because HS from bovine kidney contains unsulfated repeating disaccharide units (GlcAβ1–4GlcNAc) as the major component (~60%), and contains the monosulfated GlcA β1–4GlcNAc (~25%) and the di- or tri-sulfated IdoAα1–4GlcNAc (~15%) (Zaia, 2009). HA, composed of non-sulfated disaccharide units, does not contain sulfate. When hydrolysis step was not included, we did not observe sulfate ions in the electropherograms for all these GAG samples (data not shown). This indicates that inorganic sulfate ion was negligible in the sample. The sulfate contents found in five GAGs were in good agreement with those calculated from sulfur contents provided by the manufacturer (Table 2).

Application to the Monitoring of Chemical Sulfation of Chondroitin Sulfate

Chemical modification of polysaccharides such as sulfation affords novel biological activity, and has been well studied (Srinivasan *et al.*, 1970; Suzuki *et al.*, 2001). We synthesized some preparations of sulfated chondroitin sulfate having different degree of sulfation by changing the amount of pyridine–sulfur

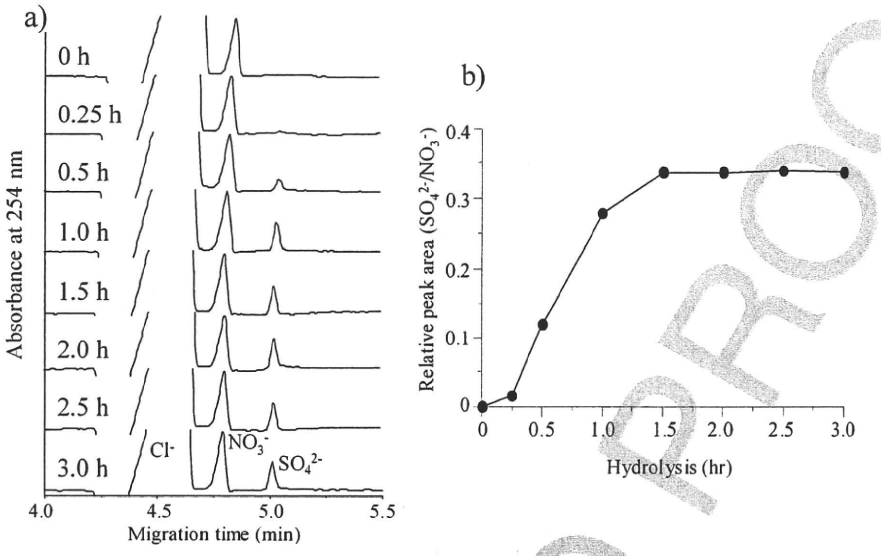

Figure 3. Time course of sulfate liberation during hydrolysis of the glucose 3-sulfate with HCl. (a) Electropherograms of the reaction mixture after hydrolysis. (b) Time course of liberation of sulfate ion from glucose 3-sulfate.

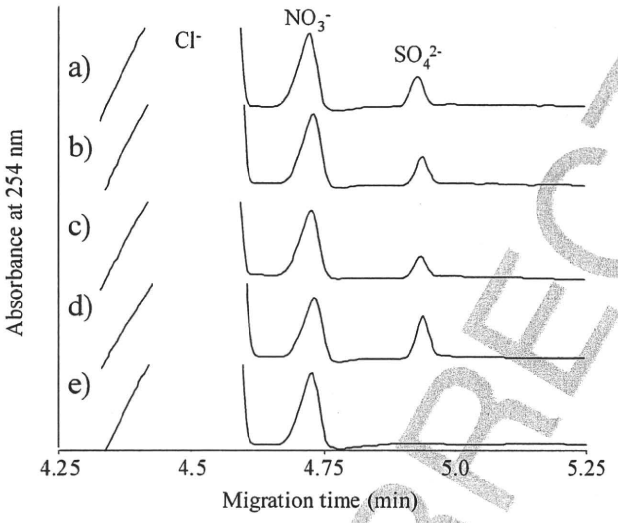

Figure 4. Determination of the sulfate content of some glycosaminoglycans. (a) Chondroitin sulfate A; (b) dermatan sulfate; (c) heparan sulfate; (d) heparin; and (e) hyaluronic acid.

Table 2. Sulfate contents of GAGs

GAGs	Total sulfate (%)[a]	
	Present method	Schoniger method[b]
Hyaluronic acid	n.d.[c]	<0.5
Chondroitin sulfate A	14.2 ± 0.5	15.0
Dermatan sulfate	15.0 ± 0.5	15.5
Heparin	25.1 ± 0.5	25.6
Heparan sulfate	11.5 ± 0.5	11.3

[a] Calculated from the dry weight of GAGs.
[b] Total sulfate contents were calculated from sufur contents provided by manufacturer.
[c] Not detected.

pyridine-sulfur trioxide complex

complex according to the method reported by Maruyama *et al.* (1998). The $PSCS_{10}$, $PSCS_{50}$, $PSCS_{100}$ and $PSCS_{250}$ were obtained from 10 mg CS using 10, 50, 100, and 250 mg pyridine–sulfur complex, respectively. Each preparation was analyzed by the present technique (Table 3).

Sulfate contents calculated from the preparations of $PSCS_{10}$, $PSCS_{50}$, $PSCS_{100}$ and $PSCS_{250}$ were 24.7, 38.5, 45.5 and 48.09%, respectively. The results showed that the amount of pyridine–surfur trioxide complex used in the reaction caused remarkable differences in the sulfate contents, and showed that the present method is useful for monitoring the degree of sulfation during chemical sulfation of oligo-/polysaccharides.

Conclusion

In the present study, we developed a simple, robust and reliable method for the determination of sulfate content in sulfated carbohydrates using CE with indirect UV detection. The background electrolyte consisting of TEA-buffered chromate with HMB is the most appropriate for the analysis of sulfate ion liberated from parent compounds after hydrolysis with HCl.

We applied the present method to the determination of sulfate content in some sulfated GAG samples such as chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate. The sulfate contents found in these GAGs were in good agreement with those obtained by conventional methods. We also applied the method to the determination of sulfate content in chemically sulfated chondroitin sulfate, and revealed the degree of sulfation.

Easy operation of the proposed technique is useful for the determination of sulfate content of sulfated oligo-/polysaccharides. The present method is suitable for routine analysis of sulfate content of carbohydrates.

Table 3. Sulfate contents of chemically sulfated chondroitin sulfate

Sample	Amount of pyridine–sufur trioxide complex	Total sulfate content (%)	Degree of sulfation (%)[b]
CSA[a]	—	14.2	27.5
$PSCS_{10}$	10 mg	24.7	47.6
$PSCS_{50}$	50 mg	38.5	74.6
$PSCS_{100}$	100 mg	45.5	88.2
$PSCS_{250}$	250 mg	48.1	93.2

[a] Chondroitin sulfate A from whale cartilage.
[b] Relative percentage to theoretical value (51.6%) of fully sulfated CSA-30mer.

References

Bourin MC and Lindahl U. Glycosaminoglycans and the regulation of blood coagulation. *Biochemistry Journal* 1993; **289**(Pt 2): 313–330.

Candelli M, Carloni E *et al.* (2000). Role of sucralfate in gastrointestinal diseases. *Panminerva Medicine* 2000; **42**(1): 55–59.

Cole DE, Evrovski J. Quantitation of sulfate and thiosulfate in clinical samples by ion chromatography. *Journal of Chromatography A* 1997; **789**(1–2): 221–232.

Fritz JS. Recent developments in the separation of inorganic and small organic ions by capillary electrophoresis. *Journal of Chromatography A* 2000; **884**(1–2): 261–275.

Giangrande PL. Fondaparinux (Arixtra): a new anticoagulant. *International Journal of Clinical Practice* 2002; **56**(8): 615–617.

Haddad PR, Doble P *et al.* Developments in sample preparation and separation techniques for the determination of inorganic ions by ion chromatography and capillary electrophoresis. *Journal of Chromatography A* 1999; **856**(1–2): 145–177.

Harakuwe AH, Haddad PR *et al.* Effect of drying on the degradation of cationic surfactants and separation performance in capillary zone electrophoresis of inorganic anions. *Journal of Chromatography A* 1999; **863**(1): 81–87.

Hattori K, Yoshida T *et al.* Synthesis of sulfonated amino-polysaccharides having anti-HIV and blood anticoagulant activities. *Carbohydrate Research* 1998; **312**(1–2): 1–8.

Hemmerich S and Rosen SD. 6′-sulfated sialyl Lewis x is a major capping group of GlyCAM-1. *Biochemistry* 1994; **33**(16): 4830–4835.

Honke K and Taniguchi N. Sulfotransferases and sulfated oligosaccharides. *Medical Research Review* 2002; **22**(6): 637–654.

Hooper LV, Manzella SM *et al.* From legumes to leukocytes: biological roles for sulfated carbohydrates. *FASEB Journal* 1996; **10**(10): 1137–1146.

Jackson RL, Busch SJ *et al.* Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiology Review* 1991; **71**(2): 481–539.

Johns C, Macka M *et al.* Enhancement of detection sensitivity for indirect photometric detection of anions and cations in capillary electrophoresis. *Electrophoresis* 2003; **24**(12–13): 2150–2167.

Kaniansky D, Masar M *et al.* Capillary electrophoresis of inorganic anions. *Journal of Chromatography A* 1999; **834**(1–2): 133–178.

Katsuraya K, Ikushima N *et al.* Synthesis of sulfated alkyl malto- and laminara-oligosaccharides with potent inhibitory effects on AIDS virus infection. *Carbohydrate Research* 1994; **260**(1): 51–61.

Katsuraya K, Nakashima H *et al.* Synthesis of sulfated oligosaccharide glycosides having high anti-HIV activity and the relationship between activity and chemical structure. *Carbohydrate Research* 1999; **315**(3–4): 234–242.

Lam SK and Ching CK. Sucralfate in clinical practice. *Journal of Gastroenterology and Hepatology* 1994; **9**(4): 401–411.

Lindahl U, Backstrom G *et al.* The antithrombin-binding sequence in heparin. Identification of an essential 6-O-sulfate group. *Journal of Biological Chemistry* 1983; **258**(16): 9826–9830.

Lopez-Ruiz B. Advances in the determination of inorganic anions by ion chromatography. *Journal of Chromatography A* 2000; **881**(1–2): 607–627.

Maruyama T, Toida T *et al.* Conformational changes and anticoagulant activity of chondroitin sulfate following its O-sulfonation. *Carbohydrate Research* 1998; **306**(1–2): 35–43.

Muzikar M, Havel J *et al.* Capillary electrophoresis determinations of trace concentrations of inorganic ions in large excess of chloride: soft modelling using artificial neural networks for optimisation of electrolyte composition. *Electrophoresis* 2003; **24**(12–13): 2252–2258.

Pacakova V, Coufal P *et al.* The importance of capillary electrophoresis, capillary electrochromatography, and ion chromatography in separations of inorganic ions. *Electrophoresis* 2003; **24**(12–13): 1883–1891.

Paull B and King M. Quantitative capillary zone electrophoresis of inorganic anions. *Electrophoresis* 2003; **24**(12–13): 1892–1934.

Roy AB and Turner J. The sulphatase of ox liver. XXIV. The glycosulphatase activity of sulphatase a. *Biochimica Biophysica Acta* 1982; **704**(2): 366–373.

Saillard S, Gareil P *et al.* Development of a capillary electrophoresis assay based on free sulfate determination for the direct monitoring of sulfoesterase activity. *Analytical Biochemistry* 1999; **275**(1): 11–21.

Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, *in vitro* and in the tissues. *FASEB Journal* 1992; **6**(9): 2639–2645.

Small DH, Mok SS *et al.* Role of proteoglycans in neural development, regeneration, and the aging brain. *Journal of Neurochemistry* 1996; **67**(3): 889–899.

Srinivasan SR, Radhakrishnamurthy B *et al.* Determination of sulfate in glycosaminoglycans by gas–liquid chromatography. *Analytical Biochemistry* 1970; **35**(2): 398–404.

Sugahara K and Kitagawa H. Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. *Current Opinion on Structural Biology* 2000; **10**(5): 518–527.

Suzuki A, Toyoda H *et al.* Preparation and inhibitory activity on hyaluronidase of fully O-sulfated hyaluro-oligosaccharides. *Glycobiology* 2001; **11**(1): 57–64.

Tadano-Aritomi K, Hikita T *et al.* Determination of lipid-bound sulfate by ion chromatography and its application to quantification of sulfolipids from kidneys of various mammalian species. *Journal of Lipid Research* 2001; **42**(10): 1604–1608.

Terho TT and Hartiala K. Method for determination of the sulfate content of glycosaminoglycans. *Analytical Biochemistry* 1971; **41**(2): 471–476.

Timerbaev AR. Recent advances and trends in capillary electrophoresis of inorganic ions. *Electrophoresis* 2002; **23**(22–23): 3884–3906.

Timerbaev AR. Capillary electrophoresis of inorganic ions: an update. *Electrophoresis* 2004; **25**(23–24): 4008–4031.

Toida T, Maruyama T *et al.* Preparation and anticoagulant activity of fully O-sulphonated glycosaminoglycans. *International Journal of Biology and Macromoles* 1999; **26**(4): 233–241.

Tsuboi S, Isogai Y *et al.* 6′-Sulfo sialyl Lex but not 6-sulfo sialyl Lex expressed on the cell surface supports L-selectin-mediated adhesion. *Journal of Biological Chemistry* 1996; **271**(44): 27213–3716.

Villanueva GB. Predictions of the secondary structure of antithrombin III and the location of the heparin-binding site. *Journal of Biological Chemistry* 1984; **259**(4): 2531–2536.

Werz DB and Seeberger PH. Carbohydrates as the next frontier in pharmaceutical research. *Chemistry* 2005; **11**(11): 3194–3206.

Wu XZ. Sulfated oligosaccharides and tumor: promoter or inhibitor? *Panminerva Medicine* 2006; **48**(1): 27–31.

Yoshida T, Yasuda Y *et al.* Synthesis of curdlan sulfates having inhibitory effects *in vitro* against AIDS viruses HIV-1 and HIV-2. *Carbohydrate Research* 1995; **276**(2): 425–436.

Zaia J. On-line separations combined with MS for analysis of glycosaminoglycans. *Mass Spectrometry Review* 2009; **28**(2): 254–272.

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4

JAPANESE REGULATORY PERSPECTIVE ON IMMUNOGENICITY

TAKAO HAYAKAWA AND AKIKO ISHII

Immunogenicity is a critical safety issue in the manufacture and clinical use of protein products and requires special attention. Several major points need to be considered when dealing with immunogenicity concerns; however, it is crucial that known product- and process-related immunogenicity factors, if any, be minimized or eliminated. The immunogenicity of a biopharmaceutical product in humans can ultimately only be assessed in clinical studies. In such clinical assessment, it is understood that an observational study of antibody formation against an active ingredient, including the desired product and product-related substances, as well as product-related impurities is very important. Moreover, the potential formation of antibodies against substances derived from the manufacturing process that may be introduced into the final product (i.e., process-related impurities) should be adequately taken into account and carefully monitored. This chapter describes some aspects of immunogenicity concerns about therapeutic protein products in Japan and discusses the practical approach to minimizing potential risks in clinical use.

4.1. INTRODUCTION

In the two decades from 1990 to 2010, more than 60 biotechnological protein products were approved as therapeutics in Japan (Table 4.1). Based on the

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.

TABLE 4.1 Biotechnology-Derived Protein Products Approved in Japan

Classification	Description	Japanese Accepted Name
Enzyme	t-PA	Alteplase, pamiteplase, monteplase
	β-Glucocelebrosidase	Imiglucerase
	α-Galactosidase	Agalsidase alfa, agalsidase beta
	α-L-Iduronidase	Laronidase
	Acid α-glucosidase	Alglucosidase alfa
	Iduronate-2-sulfatase	Idursulfase
	N-Acetylgalactosamine 4-sulfatase	Galsulfase
Plasma protein	Factor VIII	Octocog alfa, rurioctocog alfa
	Factor VII (activated)	Eptacog alfa (activated)
	Thrombomodulin	Thrombomodulin alfa
	Albumin	Human serum albumin
Hormone	Insulin	Insulin human, insulin lispro, insulin aspart, insulin glargine, insulin detemir, insulin glulisine
	Growth hormone	Somatropin
	Pegylated growth hormone	Pegvisomant
	Somatomedin C	Mecasermin
	Atrial natriuretic peptide	Carperitide
	Glucagon	Glucagon
	Follicle stimulating hormone	Follitropin alfa, follitropin beta
Cytokine	G-CSF	Filgrastim, lenograstim, nartograstim
	IL-2	Celmoleukin, teceleukin
	bFGF	Trafermin
	Erythropoietin	Epoetin alfa, epoetin beta, darbepoetin alfa
	Interferon alpha	Interferon alfa (NAMALWA[a]), interferon alfa (BALL-1[a]), interferon alfa-2b, interferon alfacon-1
	Interferon beta	Interferon beta[a], interferon beta-1a, interferon beta-1b
	Interferon gamma	Interferon gamma-1a, interferon gamma-n1
	Pegylated interferon alpha	Peginterferon alfa-2a, peginterferon alfa-2b
Monoclonal antibody	Mouse anti-CD3 antibody	Muromonab-CD3[a]
	Mouse anti-CD20 antibody conjugated with tiuxetan	Ibritumomab tiuxetan
	Chimeric anti-CD20 antibody	Rituximab
	Chimeric anti-TNF-α antibody	Infliximab

TABLE 4.1 (*Continued*)

Classification	Description	Japanese Accepted Name
	Chimeric anti-CD25 antibody	Basiliximab
	Chimeric anti-EGFR antibody	Cetuximab
	Humanized anti-HER2 antibody	Trastuzumab
	Humanized anti-RS virus antibody	Palivizumab
	Humanized anti-IL6R antibody	Tocilizumab
	Humanized anti-CD33 antibody conjugated with calicheamicin	Gemtuzumab ozogamicin
	Humanized anti-IgE antibody	Omalizumab
	Humanized anti-VEGF antibody	Bevacizumab
	Humanized anti-VEGF antibody fragment	Ranibizumab
	Humanized anti-TNF-α antibody	Adalimumab
Fusion protein	TNF receptor fused with Fc	Etanercept

[a]Cell-derived nonrecombinant protein.

data from clinical applications of protein products in Japan and other countries, it is known that all protein products are immunogenic, even if the products have full human sequences [1–3]. The typical examples in which even human proteins exhibit immunogenicity include recombinant human insulins and interferons (IFNs) [4]. Interestingly, it is known of human insulin that the deviation of one to three amino acid residues or variants generated during manufacture or storage seriously affect their immunogenicity [5, 6]. Both recombinant IFN-α2b and IFN-α2a are subtypes of human IFN that differ at only one amino acid residue, and yet exhibit great differences in immunogenicity [7]. Factors other than protein structure can also cause differences in immunogenicity. For example, the immunogenicity of a glycoprotein such as IFN-β can be enhanced by the loss of sugar chains [8–10]. Although the mechanisms by which antibodies are formed against therapeutic human protein products are not fully elucidated, risk factors related to the immunogenicity of these products have been revealed. Both product- and patient-related factors are involved in antibody formation against product components [11].

Since therapeutic protein products often contain detectable amounts of process-related impurities, immunogenicity and adjuvant activity of process-related impurities may need to be considered, in addition to the concerns about antibody formation against active ingredients (including the desired product and product-related substances). For example, host-cell proteins and lipopolysaccharides derived from cell substrates, components of the medium used for cell culture, antibodies leaked from the column used in purification processes, and reagents used in other processes should be taken into account.

Currently, there is no official guideline in Japan focusing on the immunogenicity of therapeutic proteins. Regulatory requirements for immunogenicity assessment of therapeutic proteins vary case by case according to the nature of the products, their production process, and quality attributes (including impurities, stability, and intended clinical use), as well as patient-related factors.

This chapter describes (1) the impact of immunogenicity on safety and efficacy of therapeutic protein products and (2) the quality attributes and other factors that affect antibody formation against the products, then (3) mentions approaches to assessing immunogenicity in nonclinical and clinical studies during drug development and in postmarketing surveillance. Based on these considerations together with Japan's unique experiences with the immunogenicity of protein products, practical approaches to minimizing the risks associated with immunogenicity are discussed. The opinions presented here are personal.

4.2. IMMUNOGENICITY OF THERAPEUTIC PROTEIN PRODUCTS—IMPACT ON SAFETY AND EFFICACY

As shown in Figure 4.1, the drug substance of protein products contains not only active ingredient but also impurities [12]. The active ingredient is composed of the desired product and product-related substances. Impurities consist of process-related and product-related substances. During storage, the active ingredient and product-related impurities may degrade, and most of these components can be immunogenic. Product-related substances and product-related impurities are molecular variants of the desired product, both of which have partially the same structure as the desired product; therefore, these components may contribute to the emergence of antibodies against the desired product. Moreover, impurities can act as adjuvants that may enhance the immunogenicity of the product [4]. As described in Sections 4.2.1 and 4.2.2, the consequences of antibody formation against the active ingredient and against the impurities are not the same.

4.2.1. Antibody Formation against Active Ingredient

When antibodies are formed against the active ingredient, the possible consequences are hypersensitivity reactions, change in the serum half-life of the

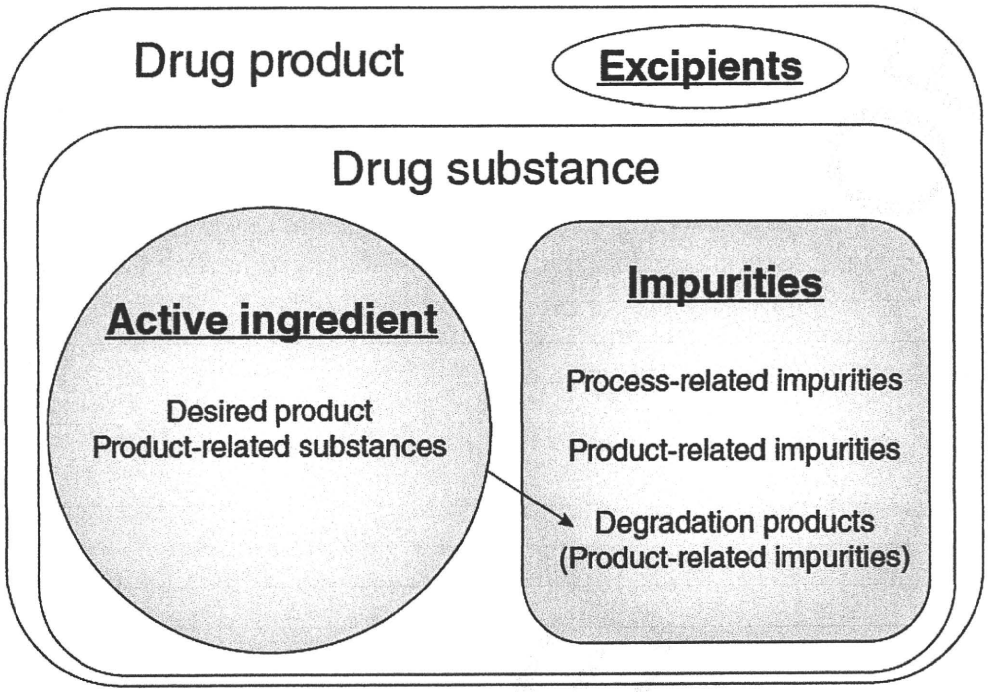

Fig. 4.1 Components of a therapeutic protein product. **Desired product** is the protein that (1) has the desired structure or (2) is expected from the DNA sequence and anticipated post-translational modification (including glycoforms) and from the intended downstream modification to produce an active biological molecule. **Product-related substances** are molecular variants of the desired product formed during manufacture and/or storage that are active and have no deleterious effects on the safety and efficacy of the drug product; these variants possess properties comparable to those of the desired product and are not considered impurities. **Product-related impurities** are molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety. **Process-related impurities** are impurities that are derived from the manufacturing process; they may be derived from cell substrates (e.g., host-cell proteins, host-cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables).

product, decrease in efficacy, or life-threatening adverse effects from neutralization of endogenous proteins by the anti-drug antibodies. The consequences after the formation of antibodies depend on the characteristics of the product and the features of the antibodies induced by the treatment.

If a product has xenogeneic amino acid sequences, the main concern is hypersensitivity reactions when the product is used repeatedly. For all kinds of protein products, hypersensitivity reactions, changes in serum half-life, or decreased efficacy may occur. If a product has the same amino acid sequence as endogenous human proteins that have critical functions and work in non-redundant pathways, such as erythropoietin or thrombopoietin, severe adverse

effects could be caused by the emergence of neutralizing antibodies that cross-react with the endogenous proteins [13–15].

Among the antibody features that affect the clinical consequences of immunogenicity, neutralizing activity and immunoglobulin (Ig) class are especially important. Emergence of neutralizing antibodies may lead to a decrease in efficacy of the product. As previously mentioned, if the neutralizing antibodies react with endogenous proteins with critical functions, not only a decrease in efficacy but also life-threatening adverse effects would occur. In cases where IgE antibodies against the product components emerge, acute hypersensitivity reactions may be the main concern.

4.2.2. Antibody Formation against Process-Related Impurities

Since process-related impurities are nonhuman materials, potential immunogenicity is inevitable. Well-known process-related impurities that may provoke antibodies are host-cell-derived proteins [16], and the possible consequences of antibody formation are hypersensitivity reactions. Some patients already have antibodies against host-cell-derived proteins or components of cell culture medium, such as albumin, that might have been formed by exposure to microorganisms or food in daily life before beginning therapy. Therefore, even during the first treatment, hypersensitivity reactions caused by antibodies against process-related impurities need to be monitored. In some cases, potential hypersensitivity of patients against specific process-related impurities should be tested before clinical application of certain biopharmaceuticals whose manufacturing process includes such reagents.

In addition to their own immunogenicity, process-related impurities can act as adjuvants for the active ingredient. These impurities may enhance antibody formation against the impurities per se or against other impurities. Therefore, to ensure the safety and efficacy of therapeutic protein products, extensive and efficient removal of such impurities should be especially important during product purification.

4.3. FACTORS AFFECTING THE IMMUNOGENICITY OF PROTEIN PRODUCTS

Humoral immune responses are predominant in the immunogenesis of protein products [17]. In general, antibodies, mainly IgM, are produced from B cells activated by the native conformational epitopes during initial exposure. When the B cells are activated by helper T cells that were activated by antigen-presenting cells that had taken up the antigens, immunoglobulin class switching occurs and the B cells then differentiate into antibody-producing plasma cells. In antigen-presenting cells, incorporated proteins are digested and the resulting peptides are presented on the cell surface by binding with major

histocompatibility antigen [MHC; human leukocyte antigen (HLA) refers to human MHC] class II molecules. It has been reported that the antibodies induced by therapeutic protein products are not only IgM but also IgG and IgE; therefore, mechanisms dependent on both B and T cells are involved in the antibody formation [17].

By genetic recombination of B-cell receptors or T-cell receptors, both B-cell and T-cell repertoires initially are highly variable in order to react with all kinds of exogenous materials [18]. As a consequence of the high variability of the receptors, the lymphocyte repertoire includes cells that react with self-antigens. The resulting self-reacting lymphocytes are deleted or induced to anergy after exposure to self-antigens via central and peripheral tolerance mechanisms [18]. However, in some situations such as onset of autoimmune disease or treatment by human protein products, self-tolerance is broken and self-reactive antibodies are produced. Considering the mechanisms of antibody formation, some features of the products such as presence of aggregates or coexistence with impurities may enhance the antibody formation pathways to overcome self-tolerance. For protein products carrying xenogeneic sequences, antibody formation is inevitable.

Factors affecting antibody formation against protein products include those discussed in the following subsections [19].

4.3.1. Product-Related Factors

4.3.1.1. Protein Primary Structure. If the protein product has xenogeneic or engineered sequences, these polypeptides are unavoidably immunogenic for humans. Mouse monoclonal antibodies, chimeric monoclonal antibodies, or engineered protein products are intrinsically immunogenic. Furthermore, even if the products have full human sequences, they can activate the human immune system. Since T-cell-mediated responses are involved in antibody formation against protein products, human proteins containing peptide sequences that bind to HLA class II molecules with high affinity (namely, T-cell epitopes) might be highly susceptible to antibody formation [20]. On the other hand, recent evidence suggests that among T-cell epitopes certain kinds of peptides have an inhibitory activity on the immune response by activating regulatory T cells [21]. Therefore, each human protein has its own potential to induce or suppress antibody formation depending on its amino acid sequences.

4.3.1.2. Sugar Chain. Many therapeutic protein products are glycoproteins. In general, glycosylation reduces the immunogenicity of proteins [4]. This may be due in part to sugar chains masking conformational or peptide epitopes. On the other hand, when protein products are produced using animal cells, the structures of sugar chains are different from those of human proteins [22]. Galactose-α-1, 3-galactose (Gal α1-3Gal) and *N*-glycolylneuraminic acid

(NeuGc) are typical nonhuman glycans that can be present in glycoprotein products [22, 23]. These nonhuman glycans are known to be antigenic for humans [24, 25]. Gal α1-3Gal is a well-known antigen that is involved in rejection of organs transplanted from pigs to humans [24]. It is now recognized that all humans have IgG antibodies specific for Gal α1-3Gal [23]. In addition, it has been reported that most normal humans have anti-NeuGc antibodies [25]. These antibodies might have been raised by exposure to food materials or microorganisms containing these nonhuman glycans.

Obvious adverse events related to these nonhuman glycans have only recently been reported. In 2008, Chung et al. [23] reported a high prevalence of hypersensitivity reactions to cetuximab, a chimeric anti–epidermal growth factor receptor (anti-EGFR) antibody product, caused by pre-existing IgE antibody against Gal α1-3Gal. Unlike most other monoclonal antibodies, cetuximab is produced in the mouse cell line SP2/0 that expresses α-1, 3-galactosyltransferase. In addition, cetuximab has N-linked oligosaccharides in its variable regions. The presence of Gal α1-3Gal on both Fab segments of cetuximab might allow the efficient cross-linking of IgE receptors on mast cells. As mentioned previously, the patients who suffered hypersensitivity reactions had anti–Gal α1-3Gal IgE prior to therapy. The reason for the presence of IgE against Gal α1-3Gal is unclear. However, these cases alert us to the fact that we should learn more about the risks associated with antibodies specific for nonhuman glycans on protein products. To our knowledge, adverse events related to NeuGc associated with the use of biotechnology-derived protein products have not been reported, although NeuGc is known as Hanganutziu–Deicher (HD) antigen, which causes "serum sickness" reactions in humans treated with animal antiserum [22, 26–28].

4.3.1.3. Molecular Variants of the Desired Products. As shown in Figure 4.1, the drug substance of protein products contains molecular variants of the desired product, that is, product-related substances and product-related impurities. These variants include aggregated, degraded, oxidized, or deamidated products. They arise during manufacture or storage. Among the variants, aggregates are well known to be immunogenic [29]. Since aggregates have repeated structure that efficiently activates B-cell receptors, they are liable to initiate immune responses [30]. In addition, aggregates enhance T-cell responses by activating antigen-presenting dendritic cells, thereby potentiating antibody formation [31]. Factors known to affect protein aggregation include protein concentration, pH, ionic strength, metal ions, organic solvent, temperature, pressure, shaking, shearing, interaction with the hydrophobic surface of containers, freeze-drying, reconstitution, and freeze–thaw cycles [32].

Even if the desired products have full human amino acid sequences, the molecular variants might constitute different epitopes from human endogenous proteins, and possibly be recognized as non-self-antigens. If the mechanisms of so-called epitope spreading are activated, antibodies against the desired product might subsequently be developed [33].

4.3.1.4. Impurities That Act as Adjuvants. Protein products usually have small amounts of process-derived impurities such as host-cell proteins, host-cell DNA, cell culture medium components, column leachables, or processing reagents. These impurities may possibly work as adjuvants for the products. In the antibody-producing pathway, there are several steps that can be potentiated by adjuvants [34, 35]; therefore, impurities of different structure could possibly work independently or synergistically to enhance antibody formation. In the well-known case of pure red cell anemia associated with subcutaneous injection of the erythropoietin Eprex, leachates from the rubber syringe stopper used for drug products containing polysorbate 80 were suggested to have adjuvant activity for the surrogate antigen ovalbumin [36].

4.3.1.5. Formulation. Excipients such as human serum albumin and sugars may interact with the active ingredients and result in abducts that might constitute new epitopes. Inadequate formulation may lead to increased aggregates, thereby enhancing product immunogenicity.

4.3.1.6. Immunomodulatory Properties of the Product. The biological activities of protein products also affect antibody formation. If a product has immunostimulatory or immunoinhibitory properties, formation of antibodies against the product might be stimulated or suppressed, respectively.

4.3.1.7. Route of Administration. Antibodies are formed more efficiently following subcutaneous or intramuscular injection than after intravenous injection. This may be because antigens are taken up more efficiently by antigen-presenting cells in subcutaneous or intramuscular tissues.

4.3.1.8. Storage Conditions and Product Handling. During storage, molecular variants of the desired product and product-related substances may increase. In addition, during handling by patients, improper storage may lead to deterioration of the sample [37].

4.3.2. Patient-Related Factors

4.3.2.1. Genetic Background (Genetic Defect, HLA Type). Patients congenitally lacking a gene do not have immunological tolerance to the corresponding gene product and therefore can readily generate antibodies to that product. In addition, it has been reported that the type of human leukocyte antigen (HLA) can affect immune responses [38]. This is because the affinity of peptides, processed from proteins incorporated in antigen-presenting cells, with HLA depends on the structure of the HLA molecules.

4.3.2.2. Immune State of the Patient. Many diseases affect the immune state of the patient. In patients with a disease such as cancer that can compromise

immunological function, antibodies are less likely to be formed by treatment with protein products.

4.3.2.3. Concomitant Treatment. Concomitant treatments that affect immune responses can also affect antibody formation against protein products. When an immunosuppressant is concomitantly used in a patient's treatment, antibody formation against the products may decrease.

4.3.2.4. Previous Exposure to Similar Proteins. If patients have been exposed to the same protein product or other products with a similar structure, for example, a chimeric antibody product, antibodies can be formed upon subsequent administration.

4.3.2.5. Age. Different age-group populations, such as children or the elderly, may show different immune responses. Thus, data obtained from a particular population cannot necessarily be extrapolated to other populations.

4.4. IMMUNOGENICITY ASSESSMENT REQUIRED IN DRUG DEVELOPMENT—CURRENT STATUS IN JAPAN

Currently there is no Japanese guideline focusing on immunogenicity issues of protein products. However, in the review process for marketing authorization, immunogenicity problems are discussed sufficiently. In application dossiers for marketing authorization, applicants are requested to describe the rationale of the study protocol and the validity of the methods for antibody detection and characterization. Based on the data obtained, the safety and potential risks associated with the immunogenicity of the product need to be explained. The kind of data required for the assessment of immunogenicity depends on the nature and intended clinical use of the product. In some cases, safety assessment related to immunogenicity is continued in postmarketing surveillance.

4.4.1. Immunogenicity Tests in Nonclinical Studies

Human protein products are xenogeneic proteins for experimental animals, and antibodies arise with high frequency. Therefore, it is well recognized that immunogenicity tests in animals are generally not predictive of a potential for product immunogenicity in humans.

Nevertheless, nonclinical evaluation of antibody formation against the product in question is almost always required. One of the principal purposes of antibody detection in nonclinical animal studies is to justify that the nonclinical studies have been conducted using relevant animal models with respect to immunological responses. Another is not necessarily to predict the immunogenicity of the product of interest in humans per se, but rather to investigate

the potential impact of the emergence of antibodies, if any, in humans on various safety- and efficacy-related parameters prior to a clinical study, as well as to support the interpretation of the nonclinical study results. Antibody responses should be characterized (e.g., titer, number of responding animals, neutralizing versus non-neutralizing), and their appearance should be correlated with any pharmacological and/or toxicological changes, where necessary and possible. Specifically, the effects of antibody formation on pharmacokinetic or pharmacodynamic parameters, the incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data [39].

There are several *in silico*, *in vitro*, and *in vivo* methods for estimating the relative immunogenicity of proteins in humans [40, 41]. These methods can be useful in the initial stages of drug development for engineered protein products in order to select a less immunogenic molecule as a new drug candidate.

In silico methods include identification of T-cell epitopes in the protein of interest that will be presented on antigen-presenting cells by binding to HLA class II molecules [20]. The number of T-cell epitope peptides and their affinity to HLA molecules are expected to correlate with immunogenicity of the protein. Recently, it was reported that some of the T-cell epitopes work as suppressors for antibody response by activating regulatory T cells [21]. Therefore, by *in silico* methods the immunogenic property of the protein is shown as a sum of T-cell epitopes that lead to the stimulation or suppression of the immune response. By *in vitro* T-cell activation assay, the potency of each T-cell epitope, identified by *in silico* experiments, to activate T cells can be evaluated. Correlation between *in silico* experimental results and clinical responses has been reported for several proteins [42]. Although there are several methods that predict the presence of B-cell epitopes, the quality of B-cell epitope predictions is considered to be too poor to be employed as a reliable tool [40].

In vivo methods using human protein transgenic mice and HLA (human MHC) transgenic mice have been reported [43, 44]. Since transgenic mice expressing human therapeutic proteins are expected to be tolerant of the relevant therapeutic human proteins, breaking of self-tolerance by human protein products can be evaluated. In HLA transgenic mice, T-cell-mediated antibody formation that is activated by human-protein-derived peptides that bind to human MHC molecules can be evaluated. Double transgenic mice expressing the human protein of interest and HLA would also be useful for evaluating the immunogenicity of human protein products. HLA transgenic mice can be used to confirm the results of *in silico* experiments by comparing the relative immunogenicity of two or more protein products carrying different amino acid sequences.

There is a possibility that *in vivo* methods may be used to evaluate comparability during the development of subsequent-entry protein products or to evaluate the consequences of a process change. In such cases, however, the

protein products to be tested have the same amino acid sequence; therefore, quality-related factors such as post-translational modifications of the product, product-related impurities, or process-related impurities would contribute to differences in immunogenicity of the products. Therefore, extensive physicochemical and biochemical analyses to reveal the quality attributes that may relate to immunogenicity would be more appropriate and more practical.

If immunogenicity tests in nonclinical studies are performed to predict immunogenicity in humans, it is necessary to thoroughly demonstrate the validity of the evaluation systems employed. However, since antibody formation depends not only on product-related factors but also on patient-related factors, we think that evaluation of immunogenicity in clinical studies is required for all new-entry protein products that will be administered repeatedly. Immunogenicity testing would be continued in postmarketing surveillance in addition to investigational clinical studies.

4.4.2. Immunogenicity Assessment in Clinical Studies and Postmarketing Surveillance

Adequate antibody detection and observation in clinical studies is most important for assessing the impact of antibody formation on safety and efficacy of the product. Immunogenicity assessment in clinical studies is necessary for all novel products except those that are used for a single administration. Even if other protein drug products with the same nonproprietary name have been approved, immunogenicity assessment may be necessary depending on the quality attributes of the drug product in question. This is because immunogenicity may depend on drug-product characteristics such as formulation as well as content and variety of product- and process-related impurities. For products that have been developed overseas, it is desirable to address any ethnic differences in the frequency of antibody formation.

In immunogenicity assessment using clinical samples, appropriateness of the antibody detection methods is the key to obtaining to reliable results. The tiered approach consisting of a screening assay, confirmatory assay, and neutralizing-antibody assay is the preferable strategy [19]. The requirements for each assay are different. For screening assays, relatively high throughput and certain detection of positive samples are required. The rationale of the criteria used for judging whether a sample is positive, for example, the cutoff value of optical density in an enzyme-linked immunosorbent assay (ELISA), should be shown. For confirmatory assays, confident judgment of the presence of antibodies is required. For neutralizing-antibody assays, methods that can adequately evaluate the biological activity of the drug should be used.

The methods commonly used for the detection of binding antibodies are ELISA, radioimmunoprecipitation (RIP) assay, electrochemiluminescent (ECL) assay, and surface plasmon resonance (SPR) assay. The advantages and disadvantages of these methods are listed in Table 4.2 [45]. For screening and confirmatory assays, ELISA and RIP would be the most commonly used

TABLE 4.2 Advantages and Disadvantages of Various Anti-Drug Antibody Assay Formats

Method	Advantage	Disadvantage
ELISA	Sensitive	May not detect early immune response (especially rapidly dissociating or low-affinity antibodies)
	Inexpensive	May be influenced by high levels of circulating drug (especially bridging format)
	Equipment readily available	
ECL	Sensitive	Equipment can be expensive
	Can be modified to respond in the presence of high levels of circulating drug	May not easily detect rapidly dissociating antibodies
SPR	Method of choice for detecting early immune response	Expensive equipment
	Antibody characterization capabilities	Generally less sensitive than RIP, ELISA, or ECL (but more sensitive for rapidly dissociating antibodies)
RIP	Sensitive	May not detect early immune response
	Inexpensive	May be influenced by high levels of circulating drug
	Equipment readily available	

Source: This table was adapted from "Assays and strategies for immunogenicity assessment" presented by Swanson SJ at the BMWP/BWP workshop on immunogenicity assessment of therapeutic proteins [45].

methods, respectively. SPR assays are useful for detecting an early immune response or characterizing antibody class. Other methods such as Western blotting can be used, if appropriate. However, there is no perfect method for antibody detection. Applicants should justify their strategies for antibody detection. It is desirable to measure not only IgG but also IgE and IgM antibodies in order to assess the possibility of hypersensitivity reactions and an early immune response, respectively. In the development of engineered protein products such as an insulin analogue, cross-reactivity of antibodies with the endogenous proteins (i.e., insulin in this case) should be investigated. If patients who received a therapy using protein products of similar structure have been found to have antibodies against the active ingredient before the study, antibody concentration or titer before the study can be used as a control for each case.

Regarding samples in which the presence of antibody was confirmed, neutralizing activity of the antibodies should be carefully examined, because the most distinctive clinical effect of anti-drug antibody formation is a decrease in efficacy of the product. Neutralizing antibodies that also inhibit the