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Monosaccharide composition analysis of pamiteplase by anion exchange chromatography with pulsed amperometric detection

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Abstract

The monosaccharides (neutral and amino sugars) of palmiteplase (recombinant modified human tissue plasminogen activator) were analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Since the palmiteplase formulation contains sucrose, it was removed by reverse-phase high-perfomance liquid chromatography (RP-HPLC) prior to analysis. Acid hydrolysis with TFA was performed at 100°C for 4 h. Fucose, glucosamine, galactose and mannose were detected by HPAEC-PAD analysis after hydrolysis. The linearity range of HPAEC-PAD analysis was 20–200 pmol ml^{-1} ($r > 0.999$) and the RSD value for repeatability was less than 7%. The recovery of each monosaccharide spiked into samples was more than 90%. The monosaccharide composition of palmiteplase suggests that it has complex-type oligosaccharides lacking in high-mannose-type oligosaccharides. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Glycoprotein; Monosaccharide composition analysis; HPAEC-PAD; Pamiteplase; YM866; Recombinant human tissue plasminogen activator

1. Introduction

Monosaccharide composition analysis of glycoproteins is one of the most important methods for the functional and biological studies of oligosaccharides. This analysis can be conducted using techniques such as high-performance liquid chromatography [1–4], gas chromatography [5] and capillary electrophoresis [6–8]. For pharmaceutical recombinant glycoproteins, monosaccharide composition analysis is critical not only for characterizing their carbohydrate structures, but also for monitoring product consistency. Especially for the latter purpose, a simple and reliable method is preferable, and a highly sensitive method is not necessarily required because sufficient quantities of samples can be obtained in the case of pharmaceutical recombinant proteins. High-performance

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anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is one of the most suitable methods for this purpose because it is quantitative and does not require monosaccharide derivatization [1]. In HPAEC-PAD analysis, monosaccharides are separated on an anion-exchange column at alkaline pH, followed by electrochemical detection of monosaccharides by pulsed amperometric detection (PAD).

Tissue plasminogen activator (t-PA) is a glycoprotein that activates fibrinolysis by converting plasminogen to plasmin. Recombinant t-PAs have been used clinically for thrombolytic therapy [9]. The high-mannose-type oligosaccharides of t-PA are critical for its clearance in vivo [9,10]. Pamiteplase (YM866) produced by Chinese hamster ovary cells (CHO cells) is a novel recombinant modified human tissue plasminogen activator (t-PA) which lacks the first kringle domain (del. 92–173) and contains a site mutation at the second kringle-light chain linkage site (^{275}Arg → Glu) [11]. Interestingly, its clearance rate from plasma in vivo is much slower than that of native t-PA [12,13].

Pharmaceutical recombinant products, including pamiteplase drug substance, often contain carbohydrate additives such as sucrose to enhance their stability. Therefore, to determine and to monitor the monosaccharide composition of such a product, it is essential to remove this carbohydrate because it interferes with the analysis. However, this removal process may degrade the accuracy or reproducibility of monosaccharide composition analysis.

The monosaccharide composition of neutral and amino sugars of pamiteplase was quantitatively analyzed by HPAEC-PAD after acid hydrolysis by trifluoroacetic acid (TFA). Since pamiteplase drug substance contained sucrose, it was removed by reverse-phase high-performance liquid chromatography (RP-HPLC) before the acid hydrolysis. The monosaccharide composition obtained suggests that pamiteplase has complex-type oligosaccharides lacking in high-mannose-type oligosaccharides. The results of linearity, repeatability and accuracy show that this method is also useful to monitor the product consistency of pamiteplase.

2. Experimental

2.1. Materials and reagents

Pamiteplase drug substance contains 1 mg ml^{-1} pamiteplase, 10% (w/v) sucrose, 3% (w/v) sodium succinate and 0.05% (w/v) Tween 80. Fucose (Fuc) was purchased from Sigma (St Louis, MO); glucosamine (GlcN), galactose (Gal), mannose (Man) and acetonitrile from Wako (Osaka, Japan); trifluoroacetic acid (TFA) from Pierce (Rockford, IL); 50% sodium hydroxide (w/w) from Fisher (Pittsburgh, PA). Ultrapure water was supplied from a Millipore Milli-Q system. The water for all eluents was degassed in a sonicator under vacuum for more than 10 min just before use. Glass tubes (10 × 75 mm) used for hydrolysis were purchased from Maruemu (Osaka, Japan), and were pretreated with 6 M HNO_3 and then with ultrapure water, followed by heat treatment at 500°C for more than 6 h. All other reagents were of analytical grade.

2.2. Removal of sucrose

Removal of sucrose was conducted using a Waters LC system consisting of a Model 626 non-metallic pump, a Model 700 autosampler and a Model 490 UV detector. Two-hundred microliters of pamiteplase drug substance was injected on a Vydac C4 column (4.6 × 150 mm; Hesperia, CA). The mobile phases used include solvent A (0.1% (v/v) TFA in water) and solvent B (0.1% TFA (v/v) in acetonitrile). The column was initially equilibrated at 30% B at a flow rate of 1 ml min^{-1}. The separation was performed by a linear gradient of 30 to 50% B for 20 min at a flow rate of 1 ml min^{-1} at ambient temperature. Detection was by UV absorbance at 280 nm. The main peak was collected in a polypropylene tube. The optical density of collected fractions was measured with a spectrophotometer at 280 nm, and the protein concentrations of the fractions were determined from the optical density and extinction coefficient (86 400 M^{-1} cm^{-1}) of pamiteplase at 280 nm according to the Beer–Lambert law.

2.3. Hydrolysis

The RP-HPLC purified fraction was transferred to a glass tube after measuring the volume and was dried with a SpeedVac vacuum concentrator. The sample was dissolved with 0.5 ml of 4 M TFA, and then hydrolyzed at 100°C for 4 h using a Waters Pico-Tag Workstation. After hydrolysis, residual TFA was removed with a SpeedVac vacuum concentrator. The dried sample was dissolved in 0.125 ml of ultrapure water before HPAEC-PAD analysis. As a reference, a mixture of 125 nmol each of Fuc, GlcN, Gal and Man was subjected to the same conditions as the hydrolysis of pamiteplase as described above.

2.4. HPAEC-PAD analysis

Monosaccharides were detected using a HPAEC-PAD system consisting of a Waters Model 626 non-metallic pump, a Waters Model 700 autosampler, a Fluid Delivery Module (Michrom BioResourses, Auburn, CA) and a Dionex PAD 2 detector with a gold working electrode. Fifty microliters of the hydrolyzed sample was injected into a Dionex Carbopac PA1 column (4 × 250 mm; Sunnyvale, CA). The separation of monosaccharides was performed at an isocratic concentration of 16 mM NaOH at a flow rate of 1 ml min^{-1} at 27°C. After isocratic elution for 20 min, the column was washed with 1 M NaOH for 20 min and then re-equilibrated with 16 mM NaOH for 15 min. The NaOH eluents were pressurized with helium during analysis to avoid carbon dioxide contamination from the air. The post-column eluent was 1 M NaOH which was delivered at a flow rate of 0.5 ml min^{-1} by helium gas pressure using the Fluid Delivery Module. The PAD conditions were as follows: $E_1 = +0.1$ V, $t_1 = 480$ ms, $E_2 = +0.6$ V, $t_2 = 120$ ms, $E_3 = -0.8$ V, $t_3 = 120$ ms. The resulting chromatographic data were integrated and calculated using Waters Millennium software.

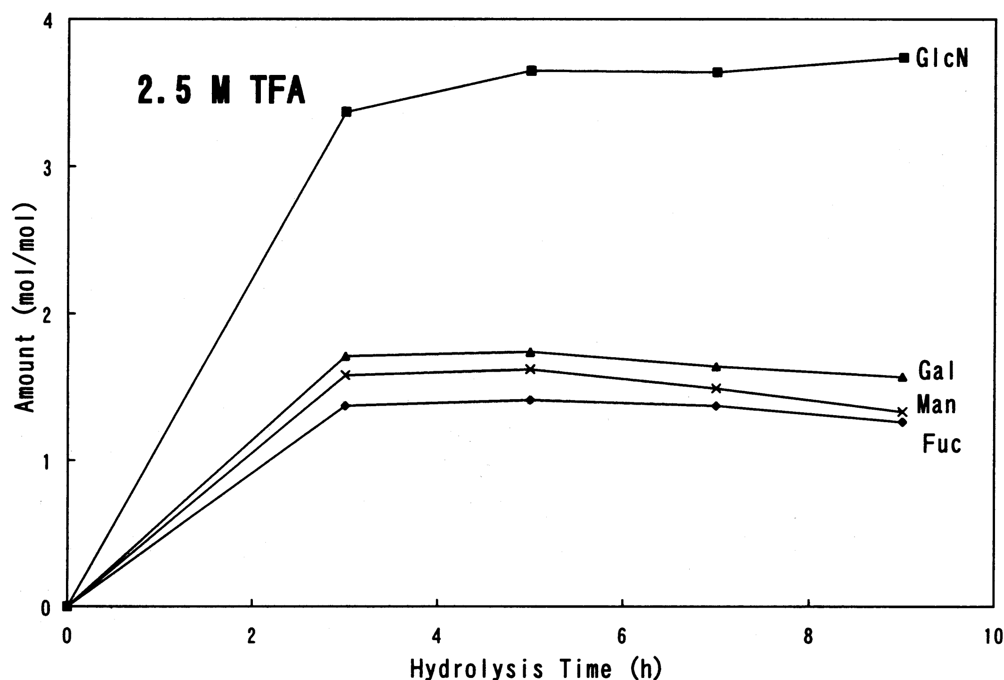

Fig. 1. Effect of TFA concentration and hydrolysis time on recovery of monosaccharides of pamiteplase. Hydrolysis was performed in 2.5 M at 100°C. Monosaccharides were determined by the HPAEC-PAD as described in Section 2.

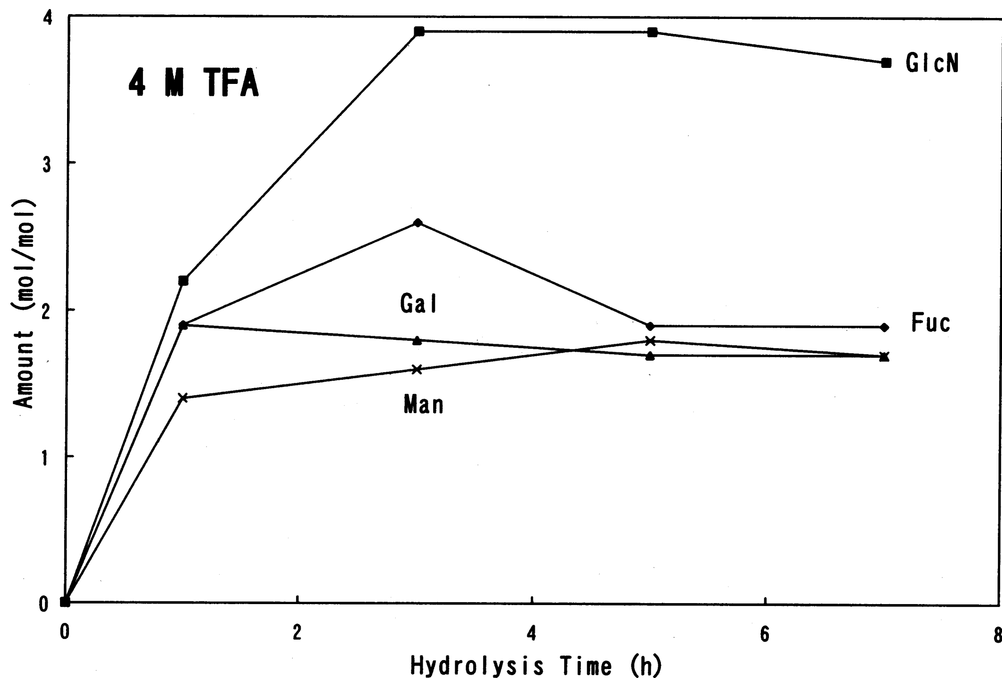

Fig. 2. Effect of TFA concentration and hydrolysis time on recovery of monosaccharides of pamiteplase. Hydrolysis was performed in 4 M TFA at 100°C. Monosaccharides were determined by the HPAEC-PAD as described in Section 2.

3. Results and discussion

3.1. Monosaccharide composition analysis

Pamiteplase drug substance contains sucrose which is converted to glucose and fructose by acid hydrolysis. The concentration of sucrose is more than 1000 times higher than the concentrations of monosaccharides derived from pamiteplase oligosaccharides. Fructose would not interfere with this monosaccharide analysis because it eluted after 20 min in this HPAEC-PAD condition (data not shown). However, glucose (Glc) would interfere with this analysis because it eluted between Gal and Man. Therefore, RP-HPLC was used to remove sucrose from pamiteplase drug substance before acid hydrolysis. Acid hydrolysis was performed using 2.5 and 4 M TFA at 100°C and the liberated monosaccharides were determined by HPAEC-PAD. Maximum recovery was observed using either 2.5 M TFA for 5–9 h (Fig. 1) or 4 M TFA for 3–5 h (Fig. 2). Because there was no significant difference in recovery between the two conditions, 4 M TFA for 4 h was selected as an acid hydrolysis condition to reduce the total analysis time.

After acid hydrolysis, the dried sample was analyzed using HPAEC-PAD. Representative chromatograms of monosaccharide standards and pamiteplase drug substance by HPAEC-PAD are shown in Fig. 3. Fuc, GlcN, Gal and Man were detected from pamiteplase drug substance. Glc observed in the chromatograms may be derived from contamination or from remaining sucrose. Although several unknown peaks which might be derived from amino acids or peptides were observed, they did not interfere with detection of Fuc, GlcN, Gal and Man.

3.2. Linearity, repeatability and accuracy

The linearity of HPAEC-PAD was determined using 20–200 pmol μl^{-1} of monosaccharide standards. The results are shown in Table 1. The correlation coefficient for each monosaccharide was more than 0.999. Repeatability was also ex-

amined for the processes of HPAEC-PAD, acid hydrolysis and sucrose removal. A monosaccharide standard was used for HPAEC-PAD repeatability, and pamiteplase drug substance for the total analysis. For repeatability of the processes from acid hydrolysis to HPAEC-PAD, a pooled main peak fraction obtained from multiple injections of a sample on the RP-HPLC was analyzed. The results are summarized in Table 2. The R.S.D. (%) of total analysis was 2–7% and that of the processes from acid hydrolysis to HPAEC-PAD was 2–3% whereas that of HPAEC-PAD was less than 1%. These results indicate that most variability for this method is

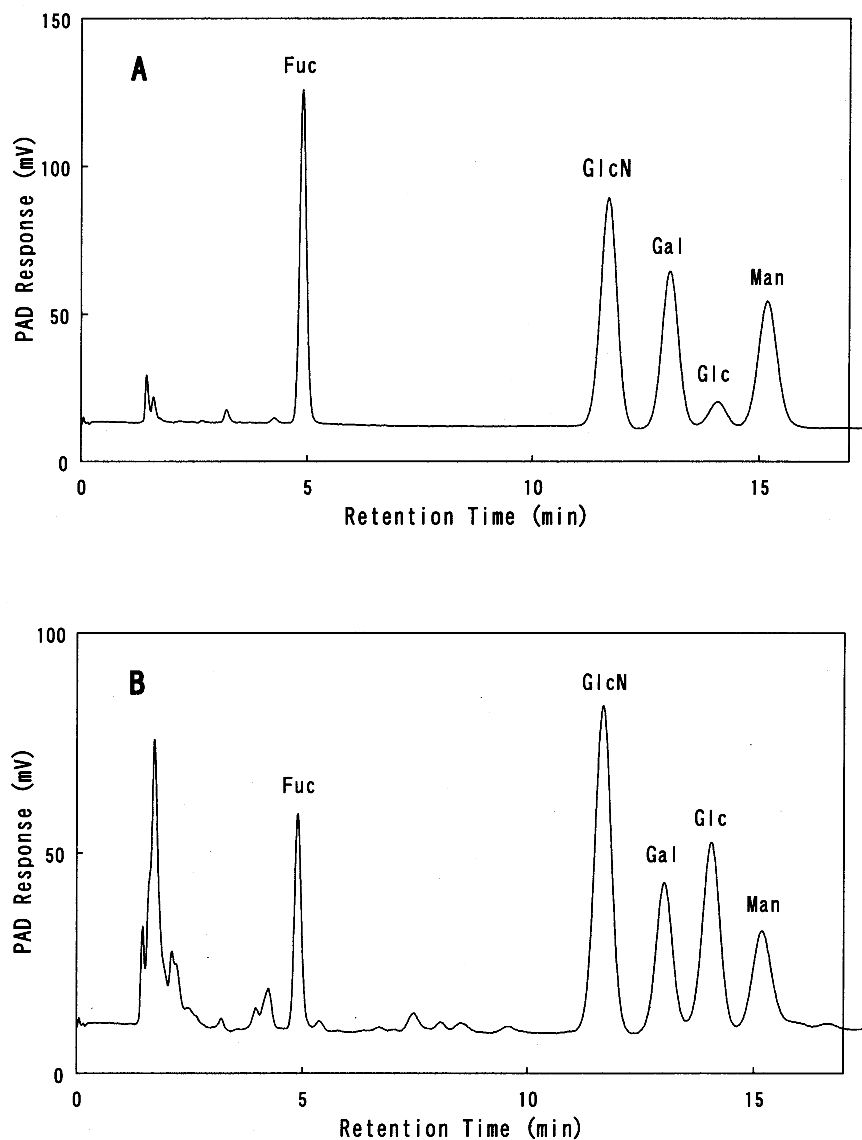

Fig. 3. Representative chromatogram of standard monosaccharides (A) or monosaccharides from pamiteplase (B) by HPAEC-PAD. Monosaccharides and pamiteplase drug substance were separated by RP-HPLC, hydrolyzed in 4 M TFA for 4 h and detected by HPAEC-PAD as described in Section 2. The amount of each standard monosaccharide was 5 nmol (A), and each monosaccharide was derived from approximately 1 nmol of pamiteplase (B).

Table 1
Linearity data for monosaccharide analysis by HPAEC-PAD[a]

	Correlation coefficient	Slope	Intercept
Fuc	>0.9999	22.7 ± 0.084	0.330 ± 0.514
GlcN	>0.9999	29.0 ± 0.130	−0.248 ± 0.797
Gal	>0.9999	22.2 ± 0.108	0.423 ± 0.662
Man	0.9995	21.7 ± 0.387	0.120 ± 2.374

[a] Analyses were performed at concentrations of 20, 50, 100, 150 and 200 pmol μl^{-1}. The results of slope and intercept are expressed as mean ± S.E.

Table 2
Repeatability data for monosaccharide analysis[a]

	R.S.D. (%)			
	Fuc	GlcN	Gal	Man
HPAEC-PAD	0.49	0.66	0.98	0.75
Hydrolysis and HPAEC-PAD	3.19	3.26	2.22	3.48
Total analysis	6.65	6.97	5.73	1.99

[a] Results were obtained from seven replicates.

Table 3
Accuracy data for monosaccharide analysis

	Fuc	GlcN	Gal	Man
Recovery (%)	94.8	99.9	100.1	89.3
	96.3	92.6	96.7	90.6
	89.2	91.9	94.0	94.8
Mean (%)	93.4	94.8	96.9	91.5
R.S.D. (%)	4.0	4.7	3.1	3.2

derived from the processes of acid hydrolysis and sucrose removal.

Recovery was examined by spiking monosaccharides into the main peak fraction. The results are shown in Table 3. Mean recovery (%) of each monosaccharide was more than 90%. The mean recovery of mannose (91.5%) was slightly less than those of other monosaccharides (93.4–96.9%). The decrease of mannose recovery might be due to interference with mannose PAD detection by lysine, which has been reported by Weitzhandler et al. [14].

3.3. Monosaccharide composition of pamiteplase

Five different lots of pamiteplase drug substance were analyzed with this method. The results are shown in Table 4. The monosaccharide compositions obtained from all five lots of drug substance were consistent. The average amounts of Fuc, GlcN, Gal and Man were 1.8, 4.3, 2.7 and 2.5 (mol/mol pamiteplase), respectively, which is quite different from those of other native t-PAs [15,16] previously reported. Specifically, the mannose content of pamiteplase was much lower than those of native t-PAs, which have high-mannose-type oligosaccharides at ^{117}Asn and complex-type ones at ^{184}Asn and ^{448}Asn [17–20]. However, the monosaccharide composition of pamiteplase was similar to that of complex-type oligosaccharides at ^{448}Asn of the genetically engineered t-PA variant [21], which contains Gln in place of ^{117}Asn and ^{184}Asn (Table 4). These results suggest that the major oligosaccharides of pamiteplase are complex-type and that pamiteplase lacks high-mannose-type oligosaccharides. This seems to be reasonable since pamiteplase lacks the ^{117}Asn in the kringle-1 domain (del. 92–173) which high-mannose-type oligosaccharides normally attach to. The lack of high-mannose-type oligosaccharides of pamiteplase may cause its slower clearance rate of pamiteplase from plasma in vivo than that of native t-PA [12,13].

The observed fucose content (1.8 mol/mol) was more than the theoretical maximum fucose content (1 mol/mol) of the fucosylated complex-type oligosaccharides. Native t-PA has an *O*-linked fucose attached to ^{61}Thr in the epidermal growth factor domain [22] and pamiteplase has a fucose at the same position (Shimizu et al., unpublished data). It is thought that the fucose measured may come from the fucose of the fucosylated complex-type oligosaccharides and the *O*-linked fucose at ^{61}Thr.

In conclusion, the monosaccharide composition of neutral and amino sugars of pamiteplase was analyzed by HPAEC-PAD after removal of sucrose by RP-HPLC and hydrolysis by TFA. In HPAEC-PAD analysis after hydrolysis, Fuc, GlcN, Gal and Man were detected. The obtained monosaccharide composition suggests that

Table 4
Monosaccharide analysis of five lots of pamiteplase drug substance

Lot	Amount (mol/mol)[a]			
	Fuc	GlcN	Gal	Man
A	1.87 ± 0.05	4.38 ± 0.16	2.84 ± 0.33	2.49 ± 0.45
B	1.76 ± 0.07	4.36 ± 0.31	2.71 ± 0.07	2.54 ± 0.13
C	1.81 ± 0.14	4.38 ± 0.26	2.75 ± 0.23	2.52 ± 0.24
D	1.74 ± 0.07	4.24 ± 0.33	2.63 ± 0.16	2.41 ± 0.21
E	1.73 ± 0.16	4.14 ± 0.31	2.59 ± 0.24	2.45 ± 0.27
Average	1.78	4.30	2.70	2.48
Ref.[b]	2.1	5.9	3.9	11.0
Ref.[c]	2.5	7.4	3.7	9.8
Ref.[d] Biantennary	1	4	2	3
Triantennary	1	5	3	3

[a] Results are expressed as mean values ± S.D. from three independent assays.
[b] The monosaccharide composition of a human t-PA reported by Taverna et al. [15].
[c] The monosaccharide composition of a human t-PA reported by Vehar et al. [16].
[d] The theoretical monosaccharide compositions of the biantennary and triantennary complex-type oligosaccharides of a genetically engineered human t-PA variant (Nimitz et al. [21]).

pamiteplase has complex-type oligosaccharides but lacks high-mannose-type oligosaccharides. The data for linearity, precision and accuracy show that this method is also useful to monitor product consistency of pamiteplase.

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References

[1] M.R. Hardy, R.R. Townsend, Y.C. Lee, Anal. Biochem. 170 (1988) 54–62.
[2] K.R. Anumula, Anal. Biochem. 220 (1994) 275–283.
[3] D. Fu, R.A. O'Neill, Anal. Biochem. 227 (1995) 377–384.
[4] H. Kwon, J. Kim, Anal. Biochem. 215 (1993) 243–252.
[5] Y. Arakawa, T. Imanari, Z. Tamura, Chem. Pharm. Bull. 24 (1976) 2032–2037.
[6] S. Honda, S. Iwase, A. Makino, S. Fujiwara, Anal. Biochem. 176 (1989) 72–77.
[7] A. Guttman, J. Chromatogr. A 763 (1997) 271–277.
[8] T. Soga, D.N. Heiger, Anal. Biochem. 261 (1998) 73–78.
[9] A. Hotchkiss, C.J. Refino, C.K. Leonard, J.V. O'Connor, C. Crowley, J. McCabe, K. Tate, G. Nakamura, D. Powers, A. Levinson, M. Mohler, M.W. Spellman, Thromb. Haemost. 60 (1991) 255–261.
[10] F. Noorman, M. Barrett-Bergshoeff, D.C. Rijken, Eur. J. Biochem. 251 (1998) 107–113.
[11] Y. Kawauchi, T. Morinaga, M. Yokota, A. Kinoshita, K. Kawamura, Y. Suzuki, M. Takayama, K. Furuichi, H. Gushima, Thromb. Haemost. 65 (1991) 1193.
[12] M. Katoh, Y. Shimizu, Y. Kawauchi, J. Ishida, M. Takayama, M. Yokota, E. Yano, T. Kawasaki, K. Katsuta, S. Yano, T. Morinaga, T. Tsuji, A. Kinoshita, Y. Gomi, T. Takemoto, K. Itoh, H. Ezoe, H. Gushima, Thromb. Haemost. 62 (1989) 542.
[13] M. Katoh, Y. Suzuki, I. Miyamato, T. Watanabe, K. Mori, H. Arakawa, H. Gushima, Thromb. Haemost. 65 (1991) 1193.
[14] M. Weitzhandler, C. Pohl, J. Rohrer, L. Narayanan, R. Slingsby, N. Avdalovic, Anal. Biochem. 241 (1996) 128–134.
[15] M. Taverna, A. Baillet, R. Werner, D. Baylocq-Ferrier, J. Chromatogr. 558 (1991) 105–114.
[16] G.A. Vehar, M.W. Spellman, B.A. Keyt, C.K. Ferguson, R.G. Keck, R.C. Chloupek, R. Harris, W.F. Bennett, S.E. Builder, W.S. Hancock, Cold Spring Harb. Symp. Quant. Biol. 51 (1986) 551–562.
[17] G. Pohl, L. Kenne, B. Nilsson, M. Einarsson, Eur. J. Biochem. 170 (1987) 69–75.
[18] M.W. Spellman, L.J. Basa, C.K. Leonard, J.A. Chakel, J.V. O'Connor, J. Biol. Chem. 264 (1989) 14100–14111.
[19] G. Pfeiffer, M. Schmidt, K.-H. Strube, R. Geyer, Eur. J. Biochem. 186 (1989) 273–286.

[20] R.B. Parekh, R.A. Dwek, J.R. Thomas, G. Opdenakker, T.W. Rademacher, A.J. Wittwer, S.C. Howard, R. Nelson, N.R. Siegel, M.G. Jennings, N.K. Harakas, J. Feder, Biochemistry 28 (1989) 7644–7662.

[21] M. Nimitz, G. Noll, E.-P. Pâques, H.S. Conradt, FEBS Lett. 271 (1990) 14–18.

[22] R.J. Harris, C.K. Leonard, A.W. Guzzetta, M.W. Spellman, Biochemistry 30 (1991) 2311–2314.