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The effect of pH, hydrogen peroxide and temperature on the stability of human monoclonal antibody[1]

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Abstract

The stability of human monoclonal antibody (C23), which is being developed as a passive immunotherapeutic agent against human cytomegalovirus, was investigated. C23 (about 2 mg ml^{-1}) was incubated under sterile conditions for 14 days in buffers with different pH values (ranging from 4–10), in hydrogen peroxide solutions with different concentrations (0.01% or 0.1%), and in saline at 8°C or 37°C. Samples were collected on days 0, 3, 7 and 14, and various physicochemical or biological methods were used to determine the changes in C23. These methods included turbidity (absorbance at 408 nm and transmittance at 580 nm), pH, size-exclusion high performance liquid chromatography (HPLC), hydroxyapatite HPLC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing, matrix-assisted laser desorption ionization time-of-flight mass spectrometry and virus neutralization assay. Using these methods, the possible degradation processes of C23 were initially characterized. Deamidation, oxidation, fragmentation, covalent cross-links and aggregation were observed as major degradation routes. These results gave useful information for the manufacturing process and quality control of C23.

Keywords: Electrophoresis; Liquid chromatography; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Monoclonal antibody; Stability; Virus neutralization assay

1. Introduction

Recently, with the advance of biotechnology, many kinds of peptide or protein drugs have been developed for clinical use. In order to evaluate the stability or degradation mechanism of these drugs accurately, suitable analytical methods, which differ from those used for low molecular weight drugs, are required. Until now, the ICH (International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use) meeting has discussed the harmonized guidelines to evaluate the qualities or stability of biological or biotechnological products. However, the detailed analytical methodology itself is not a main item of discussion. For the purpose of supplying biotechnological drug products of good quality, it is also important to develop more excellent methods for evaluating the stability of peptides or proteins produced by hybridoma or recombinant DNA technology. In the case of monoclonal antibodies, some reports have been presented [1–6].

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Table 1
C23 monomer content, CMV neutralization capacity of C23, absorbance at 408 nm and transmittance at 508 nm after 14 days storage at 8°C or 37°C at different pH or different concentrations of hydrogen peroxide

Storage	C23 monomer content (%)	CMV neutralization capacity of C23 (%)	A_{408}	$\%T_{580}$
pH 4, 8°C	99.1	138	0.001	99.8
pH 5, 8°C	99.5	132	0.009	98.6
pH 6, 8°C	100.0	130	0.000	100.0
pH 7.4, 8°C	100.5	123	0.008	98.7
pH 8, 8°C	100.9	153	0.002	99.5
pH 10, 8°C	99.1	150	0.003	99.5
0.01% H_2O_2, 8°C	99.5	67	0.005	99.5
0.1% H_2O_2, 8°C	90.5	78	0.008	99.1
Saline, 8°C	100.9	138	0.009	99.2
pH 4, 37°C	93.5	104	0.004	99.4
pH 5, 37°C	97.2	110	0.008	99.0
pH 6, 37°C	99.1	107	0.010	98.4
pH 7.4, 37°C	98.6	52	0.010	98.4
pH 8, 37°C	98.2	122	0.004	99.5
pH 10, 37°C	84.5	19	0.007	99.2
0.01% H_2O_2, 37°C	84.4	78	0.012	98.4
0.1% H_2O_2, 37°C	55.9	83	0.019	97.8
Saline, 37°C	99.1	54	0.001	99.7

In this study, with a view to contributing additional information on the stability assessment methods, the degradation processes of C23, which had been derived using hybridoma technology, were estimated using the latest developed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF-MS) technique as well as various conventional physiochemical or biological methods.

2. Materials and methods

2.1. C23 monoclonal antibody

Hybridoma cell line C23, producing a human monoclonal antibody (kappa light chain), was used as the source for these studies. This cell line C23 was cultivated and the resultant C23 was purified as described in a previous study [7].

2.2. Test condition

C23 was adjusted under sterile conditions to about 2 mg ml^{-1} using 0.100 M acetate buffer (pH 4 and 5), 0.080 M phosphate buffer (pH 6, 7.4 and 8), 0.080 M borate buffer (pH 10), hydrogen peroxide solution (0.01% or 0.1% final concentrations), and saline for reference. The molarity of the buffers and the hydrogen peroxide solutions was adjusted to ≈ 0.154 M by adding sodium chloride. These buffers and solutions were incubated for 14 days at 8°C or 37°C. Samples were collected after 0, 3, 7 and 14 days.

2.3. Measurement of turbidity and pH

Absorbance at 408 nm and transmittance at 580 nm were measured using a UV-2200 absorbance

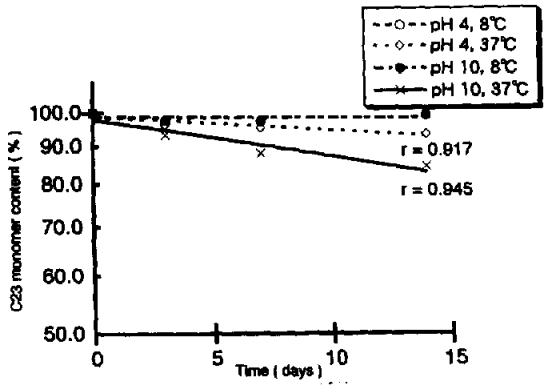

Fig. 1. Stability of C23 at pH 4 or pH 10 as a function of time.

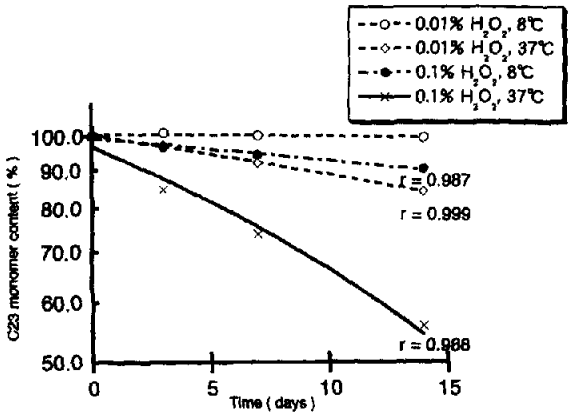

Fig. 2. Stability of C23 in the presence of hydrogen peroxide as a function of time.

spectrometer (Shimadzu, Kyoto, Japan). The pH was measured using a F-15 pH meter (Horiba, Kyoto, Japan).

2.4. Size-exclusion HPLC (SE-HPLC)

SE-HPLC was isocratically performed on two tandem prepacked TSKgel G3000SWXL columns (300 × 7.8 mm^2, Tosoh, Tokyo, Japan) with spectrophotometric detection at 280 nm. An aqueous mobile phase consisting of acetate buffer (pH 5.0; 0.050 M) with sodium sulfate (0.2 N) at a flow rate of 0.5 ml min^{-1} was used.

2.5. Hydroxyapatite HPLC (HA-HPLC)

Samples (approximately 5 μg) were loaded onto a packed TSKgel HA-1000 column (75 × 7.5 mm^2, Tosoh, Tokyo, Japan), equilibrated with diammonium hydrogenphosphate (0.100 M) containing ammonium (0.020 M) (mobile phase A) and ammonium dihydrogenphosphate (0.100 M) (mobile phase B) (30:70, v/v), and eluted with gradient increase of mobile phase B to mobile phase A–mobile phase B (80:20, v/v) over 30 min, followed by isocratic elution with mobile phase A–mobile phase B (80:20, v/v) for 15 min at a flow rate of 0.5 ml min^{-1}. Elution was monitored by absorbance at 280 nm. After analysis, the column was washed with diammonium hydrogenphosphate (0.500 M) for 30 min.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Samples (approximately 5 μg) were analyzed with or without the addition of 2-mercaptoethanol by SDS–PAGE on 4–20% acrylamide linear gradient Multi Gel (Daiichi Pure Chemicals

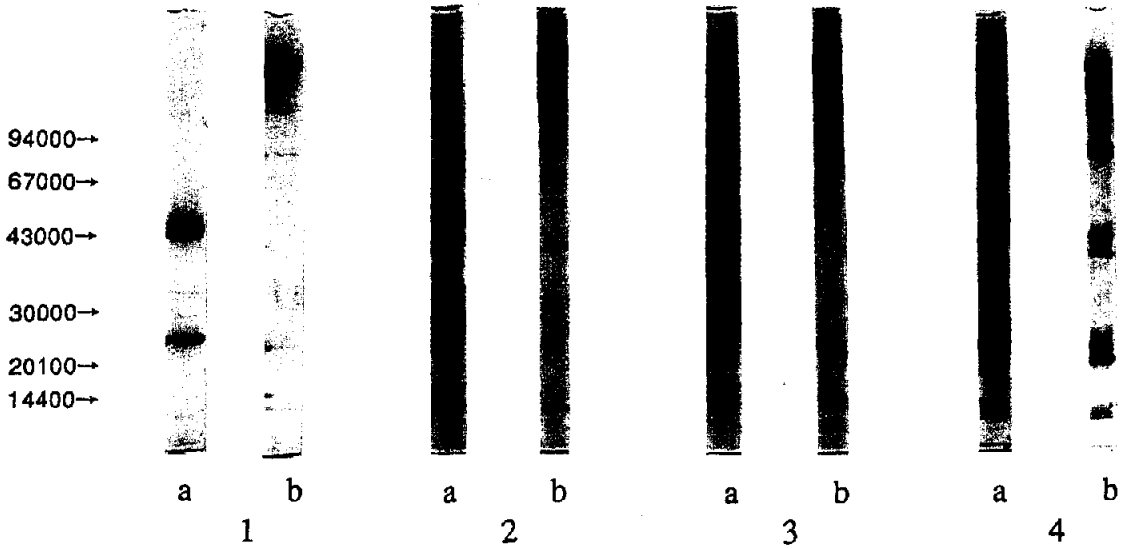

Fig. 3. Coomassie Blue-stained SDS–PAGE patterns of C23. Lanes 1: incubated at 8°C in saline for reference. Lanes 2: incubated at 37°C at pH 4. Lanes 3: incubated at 37°C at pH 10. Lanes 4: incubated at 37°C in the presence of 0.1% H_2O_2. Electrophoresis was carried out under reducing (a) and non-reducing (b) conditions.

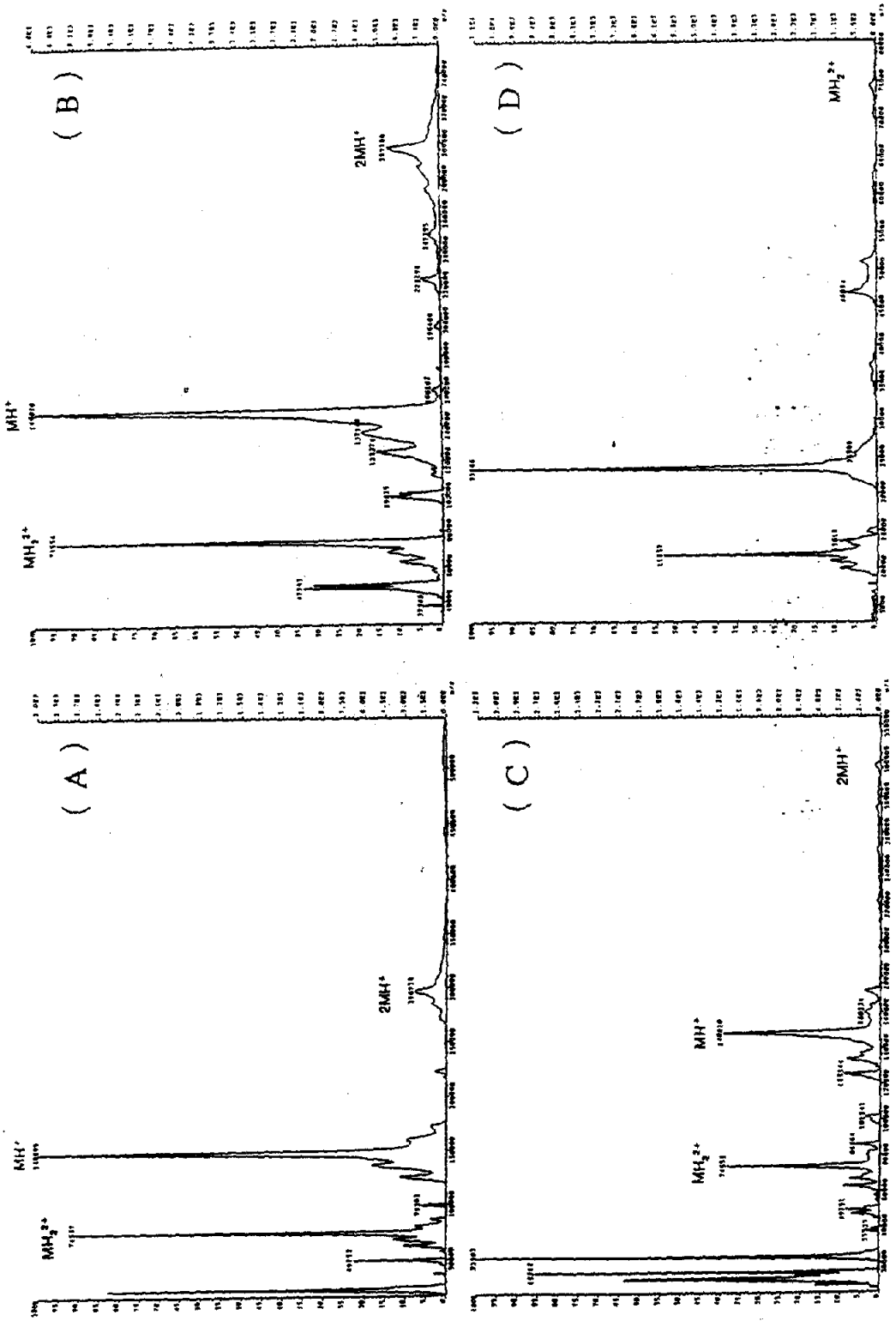

Fig. 4. Representative MALDI–TOF-MS spectra of C23 incubated at 8°C in saline for reference (A), at 37°C at pH 4 (B), at 37°C at pH 10 (C), and at 37°C in the presence of 0.1% H_2O_2 (D). In addition to the monoprotonated species and the dimer, some multiply-charged ions are observed.

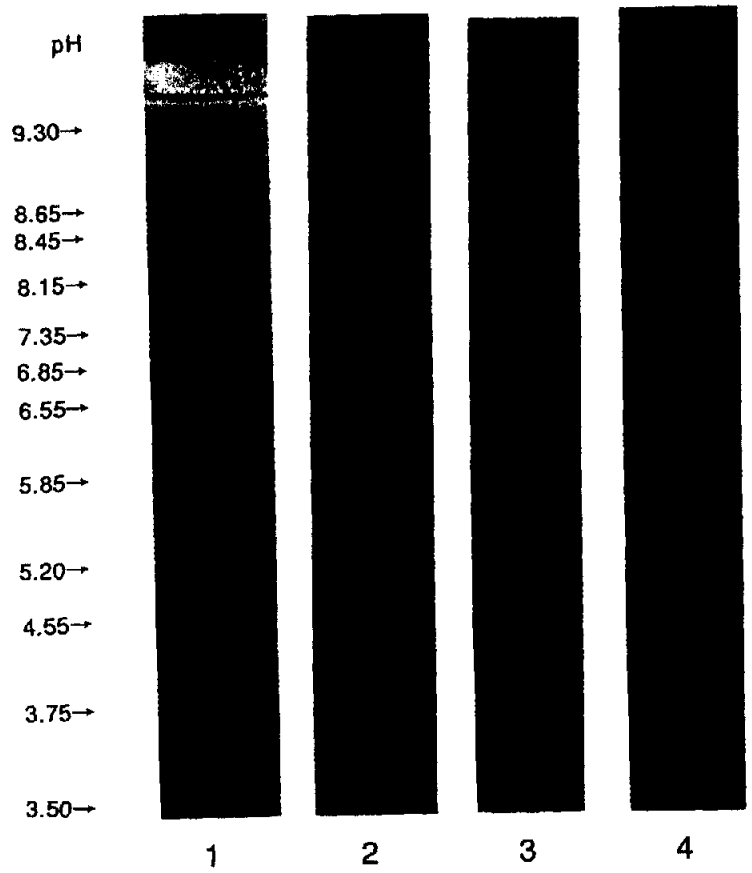

Fig. 5. Coomassie Blue-stained IEF patterns of C23. Lane 1: incubated at 8°C in saline for reference. Lane 2: incubated at 37°C at pH 4. Lane 3: incubated at 37°C at pH 10. Lane 4: incubated at 37°C in the presence of 0.1% H_2O_2.

Co., Ltd., Tokyo, Japan) in 0.3% Tris–1.4% glycine buffer containing 0.1% SDS. A constant current of 40 mA per each gel cassette was supplied over 60 min [8]. The molecular weights of the bands on Coomassie Blue-stained gels were estimated by comparison of the relative mobilities using an LMW calibration kit (Pharmacia, Uppsala, Sweden).

2.7. Isoelectric focusing (IEF)

IEF was performed in an Ampholine PAG Plate (pH 3.5–9.5) (Pharmacia, Uppsala, Sweden) with a Pharmacaia Multiphor II flat bed apparatus thermostatically maintained at 10°C. Samples (approximately 40 μg) were, applied at the center of the gel, prefocused at 100 Vh and focused at 1250 Vh. Sodium hydroxide (1 M) was used as the catholyte, and phosphoric acid (1 M) as the anolyte. Gels were fixed and stained with Coo-massie Blue according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The isoelectric points (pIs) of the bands were determined using a Broad pI Calibration Kit (pH 3–10) (Pharmacia, Uppsala, Sweden).

2.8. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Samples were prepared by mixing 1 μl (approximately 4 pmol) with sinapinic acid. MALDI–TOF-MS on a VG TofSpec E using linear mode was kindly performed by Jasco International Co., Ltd.

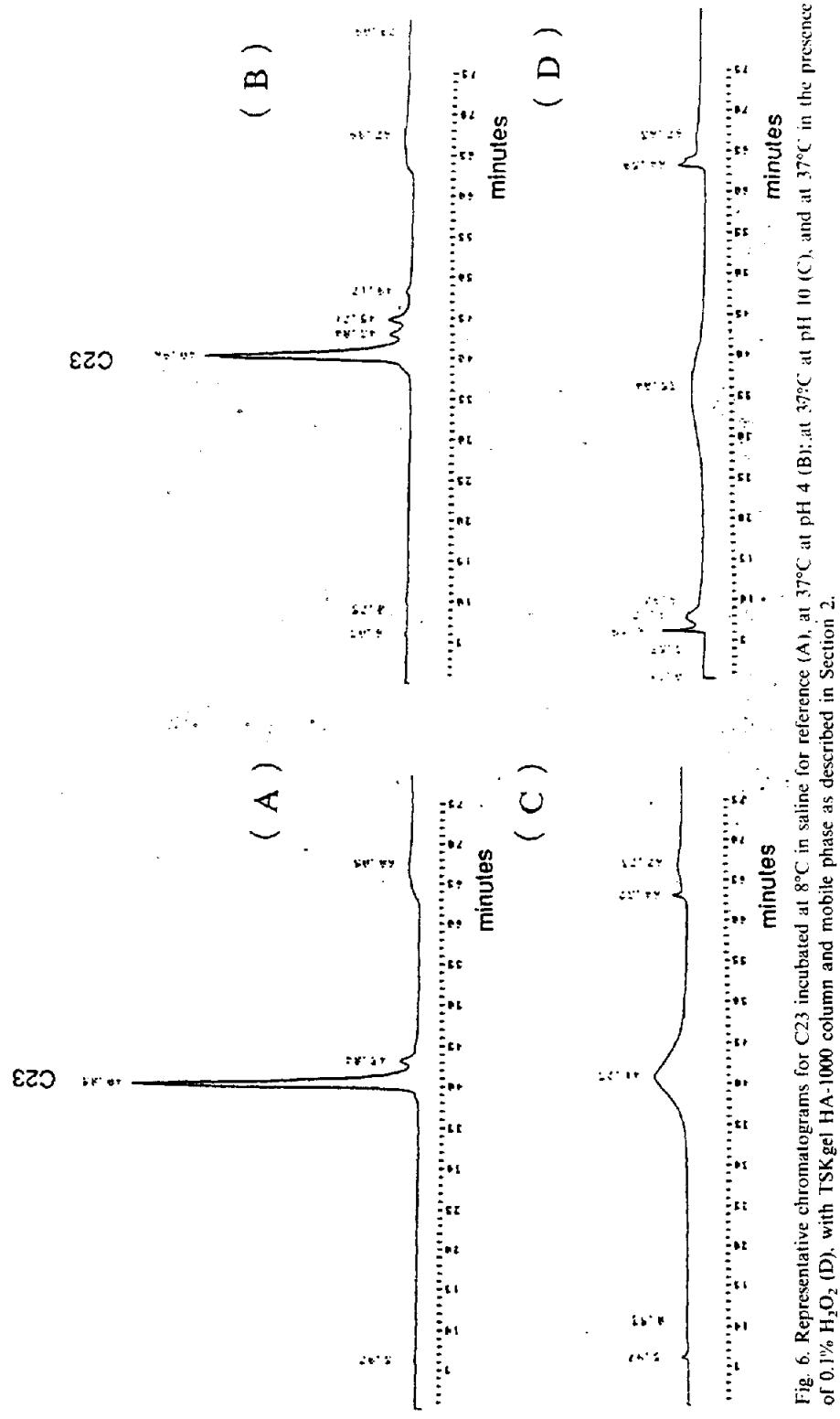

Fig. 6. Representative chromatograms for C23 incubated at 8°C in saline for reference (A), at 37°C at pH 4 (B); at 37°C at pH 10 (C), and at 37°C in the presence of 0.1% H_2O_2 (D), with TSKgel HA-1000 column and mobile phase as described in Section 2.

2.9. Virus neutralization assay

The neutralization capacity of C23 was assayed by the method described in a previous study [7].

3. Results and discussion

The methods of SE-HPLC and virus neutralization assay had been validated prior to the stability studies. The criteria for the SE-HPLC method validation were precision and linearity of the response. A relative standard deviation (RSD) of 2.9% was determined by the injection of 10 diluted samples. Calibration curves were linear ($r = 0.999$) within the working concentration range of nearly 0.5–4.0 mg ml^{-1}. In contrast, the virus neutralization assay validation was performed for precision only. An RSD of 16% was determined using seven assays.

Table 1 shows the C23 monomer content measured using SE-HPLC and the cytomegalovirus (CMV) neutralization capacity of C23 after 14 days storage at 8°C or 37°C at different pH values (ranging from 4–10) and at different concentrations of hydrogen peroxide (0.01% or 0.1%). Under both a basic condition and an oxidative condition, the virus neutralizing capacity markedly decreased. In addition, semilogarithmic plots for C23 monomer content vs time at pH 4 or pH 10 and in the presence of hydrogen peroxide are shown in Figs. 1 and 2 respectively. Under the conditions of inducing distinct degradations, the correlation coefficients were more than 0.9 (see Figs. 1 and 2). It was suggested that, if the main degradation processes of C23 were chemical reactions, the observed degradation reaction rates might approximately follow first-order kinetics [9].

Fig. 3 shows the representative Coomassie Blue-stained SDS-PAGE patterns performed under reducing or non-reducing conditions. At acidic pH, the formation of fragments was observed. At basic pH, the formation of aggregates as well as fragments was observed. In the presence of hydrogen peroxide, the formation of both fragments and aggregates was observed. Considering the results of absorbance at 408 nm and transmittance at 580 nm (see Table 1), the aggregates should be soluble under a basic pH condition and in the presence of hydrogen peroxide. In addition, the reduced samples exhibited bands with apparent molecular weights higher than that of the light chains in the case of treatment with buffer adjusted to pH 4 or pH 10 and hydrogen peroxide, and higher than that of the heavy chains in the case of treatment with buffer adjusted to pH 10 and hydrogen peroxide, as shown in Fig. 3. It was suggested that intramolecular covalent bonds between the constituent chains should be formed under an acidic pH condition, a basic pH condition and in the presence of hydrogen peroxide.

The representative MALDI–TOF-MS spectra are shown in Fig. 4. In the MALDI–TOF-MS system, all components may not be ionized; some multiply charged ions and some metastable ions might be observed in a similar manner as SDS–PAGE results accompanied by artificial bands. Therefore, one must be cautious about the explanation of MALDI–TOF-MS spectra. Nevertheless, when compared to SDS–PAGE, MALDI–TOF-MS had higher resolution and excellent accuracy. The results of both MALDI–TOF-MS and SDS–PAGE suggested that the linkage portions between the loop domains, which were composed of about 110 amino acids, could be sensitive to hydrolysis.

The typical IEF patterns are shown in Fig. 5 and the typical chromatograms of HA-HPLC in Fig. 6. Because the separation principle of HA-HPLC was mainly based on polynegative ion exchange, samples with an acidic pI value had faster retention times than those with a basic pI value under the analytical conditions described in Section 2. Comparing Fig. 5 with Fig. 6, the IEF patterns are nearly similar to the chromatograms using the HA-HPLC system, although IEF was better than HA-HPLC in terms of resolution. At acidic pH, the appearance of a discrete band with a basic isoelectric point (pI) was observed. At basic pH, a dramatic acidic pI shift was observed. In the presence of hydrogen peroxide, the pI shift was accompanied by the appearance of three bands with basic pI values and by a dramatic acidic shift. The appearance of discrete bands at basic pI may result from the formation of FAB fragments [1] and/or the removel of sialic acids [4]. The dramatic

acidic pI shifts can be assumed to mainly indicate the occurrence of deamidation under a basic pH condition and the occurrence of oxidation in the presence of hydrogen peroxide [10]. After 14 days storage in the presence of 0.1% hydrogen peroxide at 37°C, the pH changed from 5.5 to 5.2, although it was constant under the other conditions.

Under all experimental conditions, the decomposition of C23 was much more enhanced at 37°C than at 8°C.

4. Conclusions

In the present study, the possible degradation processes of C23 were initially investigated. The main degradation processes should be fragmentation and covalent cross-links at acidic pH, deamidation, fragmentation, covalent cross-links and aggregation at basic pH, and oxidation, fragmentation, covalent cross-links and aggregation in the presence of hydrogen peroxide. The results in this study indicated that MALDI–TOF-MS, as well as conventional methods, such as chromatography, electrophoresis, etc., can be used for investigating the degradation processes of monoclonal antibodies or other proteins.

References

[1] W. Jiskoot, E.C. Beuvery, A.A.M. de Koning, J.N. Herron and D.J.A. Crommelin, Pharm. Res., 7 (1990) 1234–1241.
[2] Y.J. Wang and R. Pearlman, Plenum Press, New York, 1993, pp. 135–158.
[3] D.J. Kroon, A. Baldwin-Ferro and P. Lalan, Pharm. Res., 9 (1992) 1386–1393.
[4] P.K. Tsai, M.W. Bruner, J.I. Irwin, C.C. Yu Ip, C.N. Oliver, R.W. Nelson, D.B. Volkin and C.R. Middaugh, Pharm. Res., 10 (1993) 1580–1586.
[5] D.K. Robinson, C.P. Chan, C. Yu Ip, P.K. Tsai, J. Tung, T.C. Seamans, A.B. Lenny, D.K. Lee, J. Irwin and M. Silberklang, Biotechnol. Bioeng., 44 (1994) 727–735.
[6] M. Paborji, N.L. Pochopin, W.P. Coppola and J.B. Bogardus, Pharm. Res., 11 (1994) 764–771.
[7] Y. Matsumoto, T. Sugano, C. Miyamoto and Y. Masuho, Biochem. Biophys. Res. Commun., 137 (1986) 273–280.
[8] U.K. Laemmli, Nature, 227 (1970) 680–685.
[9] S. Yoshioka, Y. Aso, K. Izutsu and T. Terao, J. Pharm. Sci., 83 (1994) 454–456.
[10] M.C. Manning, K. Patel and R.T. Borchardt, Pharm. Res., 6 (1989) 903–918.