Capillary electrophoresis and high-performance anion exchange chromatography for monitoring caseinoglycomacropeptide sialylation

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Abstract

Capillary zone electrophoresis was applied to separate caseinoglycomacropeptide glycoforms and characterize microheterogeneity of the glycopeptide. Particular attention was paid to the sialic acid content in caseinoglycomacropeptide obtained through different manufacturing processes. A chemometric approach was used to simultaneously study effects of acid concentration, hydrolysis time and temperature on sialic acid release from caseinoglycomacropeptide. Hydrolysis conditions that maximize sialic acid release were chosen. Sialic acid was determined using high performance anion exchange chromatography coupled with pulsed amperometric detection. Results were compared to those obtained by alternative techniques, such as colorimetric and enzymatic methods. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Recent progress in protein chemistry and biotechnology have induced industrial production of bioactive glycoproteins. Because various glycoproteins present great potential as drugs, there is a growing demand for efficient analytical methods to analyze these compounds and their glycoforms and elucidate the relation between biological activity and the carbohydrate structure of a glycoprotein.

Among the different caseins present in milk, κ-casein is the primary substrate of chymosin (Rennin, EC 3.4.23.4). This enzyme cleaves the peptide bond Phe105–Met106, yielding a N-terminal fragment (para κ-casein; residues 1–105), which remains with coagulated caseins, and a C-terminal fragment (a soluble caseinoglyco-macropeptide (CGMP); residues 106–169, $M_r \cong 7000$) which is recovered in the whey [1]. CGMP is a heterogeneous compound. In fact, it

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consists of a number of glycoforms with an identical peptidic backbone (except for some amino acids between genetic variants), but differ with respect to the structure, location and incidence of individual oligosaccharides. Several physiological and biological functions of CGMP have already been reported [2–5].

CGMP contains all carbohydrates originally present in κ-casein. Different *O*-glycosylation sites as well as carbohydrate chains made of one or more of the *N*-acetylneuraminic acid (NANA), galactose (Gal), *N*-acetylgalactosamine (Gal NAc) residues have been identified. A majority of carbohydrate chains contains NANA as a terminal residue α_{2-3} or α_{2-6} linked, respectively to Gal-NAc or Gal. Sialic acid was found particularly important for biological and pharmacological activity of glycoproteins and in some cases, its loss causes reduced activity [6]. Therefore, the importance of sialic acid in glycoproteins emphasizes the need to determine its amount. To further explore the biological functions of sialic acid, it is necessary to release and separate this compound from biological materials.

Release of sialic acid from glycoproteins is generally achieved by either enzymatic methods using *N*-acetyl-neuraminidase [7] or chemical hydrolysis [8]. The latter is performed by acid hydrolysis under soft conditions and typically requires 50–100 mM trifluoroacetic acid (TFA) or hydrochloric acid (HCl) heated at 80°C for 1 h [9]. However, these conditions have to be optimized for each glycoprotein to avoid decomposition or partial sialic acid release. The most important parameters are hydrolysis time and temperature as well as acid concentration. Analysis of the sialic acid released can be done with several analytical techniques including spectrophotometry [10], gas chromatography (GC) [11], high-performance liquid chromatography (HPLC) with spectrophotometric [12] or fluorimetric [13] detection, and more recently capillary electrophoresis (CE) [14]. However, due to its high selectivity and sensitivity, high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD) has been shown to be the method of choice for the analysis of carbo-

hydrates [15]. Indeed, this method does not require derivatization of released monosaccharides, since these molecules are directly detected by the amperometric detector.

This paper describes the use of capillary electrophoresis for rapid assessment of CGMP heterogeneity. Migration behaviour of CGMP glycoforms is directly linked to the number of sialic acids at the peripheral part of oligosaccharides. Chemical hydrolysis of sialic acid in CGMP is optimized using a central composite design. Once the optimized conditions were determined, sialic acid was analysed with HPAEC–PAD. Performances of the analytical method in terms of selectivity, reproducibility, linearity, sensitivity and accuracy are also presented. Finally, the method is applied to determine sialic acid content in CGMPs obtained through different manufacturing processes, as well as in various batches yielded by the same procedure. Results obtained are also compared to alternative techniques such as colorimetric or enzymatic methods.

2. Experimental

2.1. Chemicals and reagents

CGMP samples were obtained from Nestec (Vers-chez-les Blancs, Switzerland). CGMP protein content is ca. 90% as determined by total nitrogen measurement and carbohydrate content is 10%. A commercial CGMP sample was purchased from Sigma (St. Louis, MO, USA). Sodium acetate and hydrochloric acid were supplied by Fluka (Buchs, Switzerland). Sodium hydroxide, 50% (w/w) was obtained from Fisher (Pittsburgh, PA). *N*-acetylneuraminic acid (NANA) and glucuronic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were analytical grade reagents. Ultrapure water obtained by a Milli-Q RG unit from Millipore (Bedford, MA, USA) was used for standard and sample preparation. All solutions and samples were filtered through a 0.45 μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Instrumentation

2.2.1. Capillary electrophoresis

Capillary zone electrophoresis was applied according to the procedure described elsewhere [16]. CE data were generated in a HP ^{3D} Capillary Electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. Use of 20 mM citrate buffer set at pH 3.5 (Fluka, Buchs, Switzerland) was found effective to achieve baseline separation of various CGMP glycoforms.

2.2.2. HPAEC–PAD

HPAEC data were generated on a Dionex DX 500 chromatography system (Sunnyvale, CA, USA) consisting of a GP50 gradient pump and an ED40 Electrochemical detector. A Dionex cell outfitted with a gold working electrode was used for all experiments. Injections were made by a Waters 717 plus autosampler (Milford, MA). Detection output was interfaced to a software program Chrom-Card (Fisons instruments, Milan, Italy) on a AST Bravo LC 4/33 computer for data handling and chromatogram generation. The HPAEC CarboPac PA10 column (250×4 mm I.D.) associated with guard column containing the same stationary phase was supplied by Dionex. Hundred millimolar NaOH and 1 M sodium acetate in 100 mM NaOH solution were used as eluents A and B, respectively. The separation gradient was 7–30% B from 0 to 10 min, 30% B from 10 to 11 min, and 30–7% B from 11 to 12 min at a flow rate of 1 ml min^{-1}, as reported elsewhere [15]. Autosampler cycle time was 20 min. Detection was performed by using standard carbohydrate waveform ($E1 = 0.05$ V, $t1 = 400$ ms, $E2 = 0.75$ V, $t2 = 200$ ms, $E3 = -0.15$ V, $t3 = 400$ ms) [17].

2.3. Standard and sample preparation

2.3.1. Standard solutions

Stock standard solutions of NANA and glucuronic acid (1 mg ml^{-1}) were prepared in water. Working standard solutions were achieved by diluting stock standard solution with water. A calibration curve reporting peak area ratio as a function of sialic acid concentration was established in the range of 5–25 µg ml^{-1}, in the presence of 15 µg ml^{-1} of glucuronic acid as internal standard.

2.3.2. CGMP samples preparation

CGMP (300 µg ml^{-1}) was dissolved in 1 ml HCl (for concentrations, see experimental model), heated at different temperatures indicated in the experimental design model, dried in SpeedVac and dissolved in 1 ml water. Internal standard was added at a concentration of 15 µg ml^{-1}. Accuracy of the method was evaluated by recovery experiments, using standard addition technique. Three different concentrations of standard sialic acid were added to CGMP samples after hydrolysis at optimal conditions (5, 10 and 15 µg ml^{-1}). Before injection, solutions were filtered through a 0.2 µm filter

2.4. Computation

Coefficients for the regression model and optimized conditions were calculated with NEMROD (LPRAI, Marseille, France) and MATLAB (version 4.2c.1) software packages. Response surfaces were drawn with Microsoft Excel (version 7.0).

3. Results and discussion

3.1. Capillary zone electrophoresis

Due to the presence of several genetic variants as well as different glycosylation and phosphorylation sites, CGMP is known to be a heterogeneous substance. In our previous investigations, capillary zone electrophoresis using an uncoated fused silica capillary [16] or a coated poly(vinyl alcohol) (PVA) capillary [18], was applied to separate CGMP and its glycoforms. Baseline separation of different CGMP subcomponents was achieved using 20 mM citrate buffer at pH 3.5. Three groups of peaks were attributed to glycosylated non-sialylated, non-glycosylated and glycosialylated fractions [16]. Order of migration was assessed according to the charge to mass ratio of
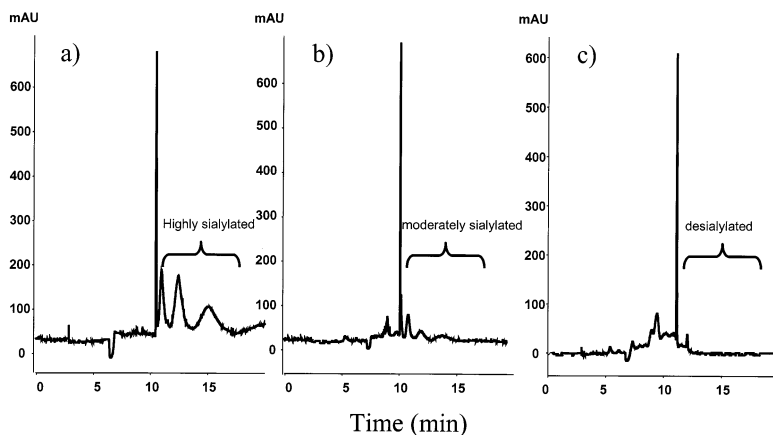
Fig. 1. Typical CGMP electropherograms: (a) highly sialylated; (b) moderately sialylated; and (c) desialylated. Operating conditions: 20 mM citrate buffer, pH 3.5, fused silica capillary (64.5/56 cm, 50 μm); applied voltage 30 kV; temperature 40°C.

these fractions. Because of the ionization of sialic acid fixed on the carbohydrate chains, glycosialylated forms have an increased negative charge.

To ascertain our assumption, three CGMP known to be highly sialylated, moderately sialylated and desialylated were analyzed under selected electrophoretic conditions. CGMP desialylation was performed by incubation with neuraminidase, an enzyme known to selectively remove sialic acid from the polysaccharide backbone. As shown in Fig. 1a–c, peaks eluting after the sharpest peak correspond well to the sialylated fractions; highly sialylated compounds exhibit higher peaks while desialylated CGMP show no peak.

A commercially available CGMP sample from Sigma (C-7278), directly prepared by chymosin action on κ-casein, was also analyzed under optimized conditions. Even if the commercial sample is obtained through a different procedure, this compound presents the same electrophoretic profile as shown in Fig. 2. From these electrophoretic results, sialic acid seems to be the origin of different charge CGMP classes.

3.2. HPAEC–PAD

With the chromatographic conditions described in the experimental section, baseline separation of sialic acid and glucuronic acid, used as internal standard, was achieved in less than 12 min as shown in Fig. 3. This method was performed without any derivatization procedure. For sialic acid release, chemical hydrolysis under mild conditions was selected due to its simplicity and low cost. However, other hydrolysis conditions are reported in the literature and thus, acid concentration, hydrolysis temperature and time have to be optimized. To perform CGMP hydrolysis, HCl was selected, as it is volatile. Moreover, among the tested acids (TFA, HCl, phosphoric acid), HCl allowed the highest sialic acid recovery.
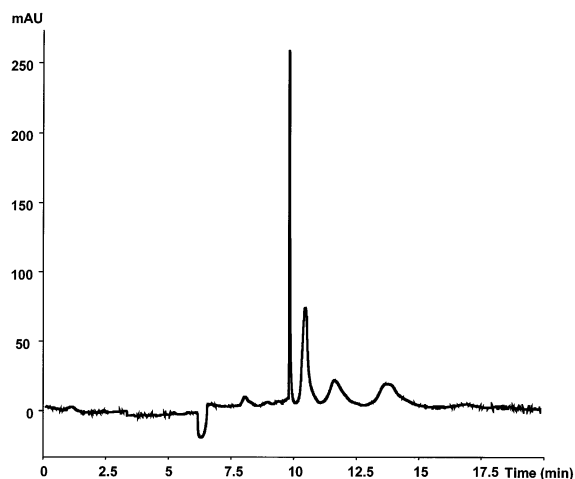

Fig. 2. Electropherogram of a commercially available CGMP (sigma). Operating conditions as in Fig. 1.
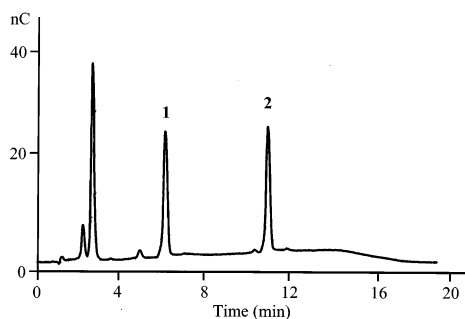
Fig. 3. Typical HPAEC–PAD profile of sialic acid released from CGMP under optimized hydrolysis conditions: (1) sialic acid; (2) glucuronic acid (internal standard). Operating conditions are given in Section 2.

3.2.1. Optimization of hydrolysis conditions

The hydrolysis method was optimized using a central composite design [19,20]. According to preliminary experiments, three relevant factors were simultaneously investigated: acid concentration (X_1), hydrolysis temperature (X_2) and hydrolysis time (X_3). Experimental factor values (levels) are summarized in Table 1. A central composite design requires 20 experiments; 2^3 points of a full factorial design, which is increased with six face centered points and six extra points performed at the center of the design. Experiments at the center were repeated six times to obtain an estimation of the experimental error. Selected experiments were randomly performed to avoid hidden effects that cannot be controlled. Sialic acid percentage was used as a response to examine the effect of each factor.

Multiple regression enables a mathematical relationship between sialic acid percentage and independent experimental variables. A central composite design provides sufficient data for fitting a second-degree expression. Coefficients of determination (R^2) and values of adjusted coefficients of determination (R_a^2) were higher than 0.94 and 0.88, respectively, indicating good predictability of the model. Residual error value of each experiment was within a range of ± 2 SD_{exp}, where SD_{exp} is the experimental standard deviation obtained through the experiments carried out at the center ($n = 6$). The quadratic regression allowed to determine optimal conditions by maximizing the percentage of sialic acid. Values of each factor in optimal conditions were 50 mM HCl, time 12 min and temperature 96°C.

For model validation, comparison was made between predicted and observed sialic acid percentage under optimized hydrolysis conditions. As a result, experimental and predicted responses were 6.84 and 6.87%, respectively, which indicate the good quality of the model.

Following validation of the model, it was also possible to draw surface responses as three-dimensional plot of two factors (HCl concentration, temperature), while keeping hydrolysis time constant at its optimal value (Fig. 4). The surface response plot shows the maximum of sialic acid release from CGMP at optimal hydrolysis conditions.

3.2.2. Validation of HPAEC–PAD method

The main objective being the routine determination of sialic acid in CGMP samples during the manufacturing process, the analytical method was validated using glucuronic acid as internal standard. Validation requires the assessment of retention time and peak area reproducibility, detector response linearity with sample concentration, sensitivity and accuracy.

Table 1
Experimental domain

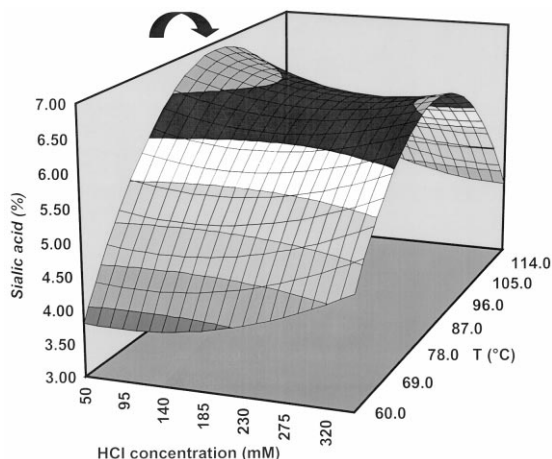| Level	$X1$	$X2$	$X3$
	Acid concentration (mM)	Hydrolysis time (min)	Hydrolysis temperature (°C)
−1	50	10	60
0	200	30	90
+1	350	50	120

Fig. 4. Surface response plot for sialic acid percentage as a function of HCl concentration and temperature. Hydrolysis time was set at optimal value (12 min).

3.2.2.1. Linearity. Detector response linearity (peak area versus concentration) was evaluated by preparing five calibration samples (5–25 µg ml^{-1}). Each sample was injected in triplicate with the internal standard. Regression curve was obtained by plotting peak area ratio (analyte peak area divided by internal standard peak area) versus concentration, using the least squares method. Linear regression correlation coefficient, R^2, was 0.999. Regression analysis data and calibration curves are shown in Table 2.

Limit of detection (LOD), defined as the lowest concentration of analyte that can be clearly detected, is estimated as three times the signal to noise ratio. LOD was determined by injecting standard solutions of various concentrations. Estimated limit of detection (Table 2) was determined as less than 0.02 µg ml^{-1}, giving a limit of quantitation (LOQ) value of less than 0.06 µg

Table 2
Regression data for calibration curve

	Sialic acid
Range [µg ml^{-1}]	5–25
Line	$y = 0.0167 + 0.0401\ x$
Correlation coefficient	0.9991
LOD [µg ml^{-1}]	0.02
LOQ [µg ml^{-1}]	0.06

Table 3
Method precision given as RSD values in%

	RSD in%
Repeatability	
Retention time	0.38
Peak area ratio	1.15
Intermediate precision	
Retention time	0.39
Peak area ratio	2.52

ml^{-1}. These values indicate the high sensitivity of the described method.

3.2.2.2. Precision. Method precision was determined by measuring repeatability and intermediate precision of retention times and peak area ratio. Repeatability (within-day precision) of the method was determined by performing replicate injections ($n = 6$) of 15 µg ml^{-1} solution containing sialic acid and internal standard. In Table 3, relative standard deviation (RSD) values are given for retention time and peak area ratio. In all cases, repeatability was better than 0.4% for retention time and 2.5% for peak area ratio, respectively.

Between-day precision was also evaluated over 3 days by performing six successive injections each day. Results (Table 3) show that reproducibility of retention time and peak area was satisfactory.

3.2.2.3. Accuracy. Accuracy of the proposed method was evaluated by recovery experiments, using the standard addition technique. Three different concentrations of standard sialic acid were added to CGMP samples after hydrolysis under optimal conditions, as shown in Table 4. Each experiment was repeated three times. Recoveries were between 98.1 and 103.7%.

3.2.3. Application

The validated method was applied to determine sialic acid content in CGMP samples obtained through different manufacturing procedures. Sialic acid was released in optimized hydrolysis conditions and then subjected to the chromatographic procedure. As shown in Table 5, different

Table 4
Accuracy determination

Dosage	Component	Labeled claim %	Amount added [μg ml^{-1}]	Amount recovered [μg ml^{-1}]	Recovery (%)	RSD (%)
CGMP	Sialic acid	6.84	5	5.18	103.7	2.04
			10	9.91	99.1	0.72
			15	14.70	98.1	1.00

Table 5
Comparison of different methods and CGMP batches

CGMP batch	Sialic acid percentage		
	Enzymatic	Colorimetric	HPAEC–PAD
501	8.23	7.45	7.75
502	6.70	6.78	6.84
506	5.09	5.00	5.13
507	4.09	4.83	4.07
601	6.03	5.66	5.44
801	6.08	5.33	5.40
804	7.49	6.25	6.23
7312	5.62	5.18	5.27
Highly sialylated CGMP			12.67
Asialylated CGMP			0.96

CGMP batches present various sialic acid content; batch 501 is highly sialylated. CGMP samples used to ascertain sialylated peaks observed in CE electropherogram were also analyzed and showed extreme sialic acid percentages (0.96 and 12.76%). Moreover, sialic acid percentages were in good agreement with results generated by two alternative colorimetric [21] and enzymatic techniques. However, while enzymatic methods are expensive, colorimetric techniques are tedious.

4. Conclusion

Capillary zone electrophoresis has been shown to be a valuable tool to monitor a glycopeptide with a charge heterogeneity presumably due to variable sialic acid content. Using a citrate buffer at pH 3.5, CGMP was separated into its different glycoforms. Migration order of investigated fragments is directly related to the increasing number of sialic acid residues. For sialic acid determination, a chemical hydrolysis under soft conditions was selected and optimum factors allowing maximum sialic acid release were determined using a central composite design. Three experimental factors were investigated: acid concentration, hydrolysis time and temperature.

Sialic acid release was monitored using high-performance anion exchange chromatography with pulsed amperometric detection. In contrast to colorimetric and enzymatic methods, the described method is rapid, selective, reliable and cost effective. Finally, the validated method was applied to determine sialic acid content in different CGMP batches and results were in good agreement with those achieved with colorimetric or enzymatic techniques.

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