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A Convenient Microscale Colorimetric Method for Terminal Galactose on Immunoglobulins

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A new approach for quantitative determination of terminal galactose (Gal) residues of immunoglobulins was developed by combining exoglycosidase digestion with the classical colorimetric estimation of reducing sugars. The ferricyanide colorimetric method was modified to increase the stability of the chromophore (Prussian blue) and adapted to determine the amount of terminal Gal residues present in immunoglobulins. The method involves the release of covalently bound Gal from immunoglobulins by *Diplococcus pneumoniae* β-D-galactosidase (specific for β(1,4) linked galactose), removal of the glycoprotein and enzyme from the reaction mixture by heat denaturation or ethanol precipitation, followed by colorimetric measurement of the released sugar using the ferricyanide assay. The ferricyanide method was modified to enhance the solubility and stability of the chromophore by increasing the concentration of aqueous sulfuric acid and sodium dodecyl sulfate (SDS). The linear range of the modified method was from approximately 11 to 111 μM Gal. Typical variation in assay results was on the order of 5%. Using the modified method, the terminal Gal content of a recombinant chimeric monoclonal antibody (anti-CD20, rIgG) expressed in Chinese hamster ovary (CHO) cells was determined and evaluated for batch-to-batch consistency. The method was used to optimize pH, time, temperature, and enzyme concentration for β-galactosidase digestion for maximal release of terminal Gal residues from rIgG.

Key Words: **galactose; colorimetric assay; ferricyanide; Prussian blue; immunoglobulins; glycoproteins; oligosaccharides; β-D-galactosidase.**

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Abbreviations used: CHO, Chinese hamster ovary cells; Ig, immunoglobulins; rIgG, recombinant immunoglobulins expressed in CHO cells; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; HPAEC-PAD, high pH anion-exchange chromatography with pulsed amperometric detection; MALDI-TOF-MS, matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry; PNGase-F, peptide-N-glycosidase F; MBP, mannose binding protein; GlcNAc, N-acetyl D-glucosamine; Gal, D-Galactose; Man, D-Mannose; GalNAc, N-acetyl D-galactosamine; SDS, sodium dodecyl sulfate.

Terminal sugar residues of the oligosaccharide side chains covalently bound to proteins and lipids influence the activity and pharmacokinetics of glycoconjugates (1). For example, Ashwell and Harford (2) have shown that asialoglycoproteins are cleared more rapidly than their sialylated counterparts because the terminal Gal residues are recognized by the asialoglycoprotein receptor present in the liver. The pattern of terminal sugars may also provide evidence about disease status (3). Sialic acids (N-acetylneuraminic acid and N-glycolylneuraminic acid) are the most common terminal sugar residues found on glycoproteins and glycolipids (4). However, Gal, Man, GlcNAc and/or GalNAc are also found as terminal sugar residues.

Fc glycans of immunoglobulins (Ig) are heterogeneous and contain mainly non-sialylated, complex biantennary structures with differing amounts of terminal Gal and GlcNAc residues. These terminal Gal and GlcNAc residues may affect Ig functions. For example, the Ig are under-galactosylated in rheumatoid arthritic patients (5). The undergalactosylated Fc glycans are recognized by the mannose binding protein (MBP) which may activate the alternative complement cascade and hence alter the normal biological functions of circulating immunoglobulins (6). Removal of terminal Gal residues from immunoglobulins was found to reduce the complement lysis activity (7). Terminal Gal residues are involved in extensive hydrogen bonding with C_H2 domain amino acid residues (8). It is possible that loss of these interactions upon removal of terminal Gal may change the orientation or dynamics of the glycan side chains, and thus alter their function.

Recombinant immunoglobulins are emerging as powerful tools for human therapy to treat life-threatening diseases such as cancer. Because of the biological significance of their oligosaccharide side chains, it is important to quantify the terminal sugar residues present in recombinant immunoglobulins. Several methods have been developed to measure the terminal sugars present in glycoproteins, both qualitatively and quantitatively (9). HPLC and CE methods involve derivatization with various UV/vis/fluorescent

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reagents. HPAEC-PAD, although it does not require derivatization, exhibits run-to-run variations due to pulsed amperometric detection (10). By combining digestion with a specific exoglycosidase and classical colorimetric assays for reducing sugars, we have developed simple, sensitive alternatives to chromatographic methods. This general approach is readily adapted to high throughput formats.

This paper describes an adaptation of the classical ferricyanide assay (11–14) which is convenient, rapid, sensitive, and highly reproducible. We achieve selectivity by using β-D-galactosidase to release terminal Galβ(1,4) residues, and perform the colorimetric assay in microtiter plates. Here we demonstrate the linearity and sensitivity of the assay, and the stability of the chromophore, using Gal as a standard reducing sugar. Using the assay, we quantified the terminal Gal residues present in a recombinant humanized chimeric monoclonal antibody to CD20 (rIgG, 15) expressed in CHO cells. The method is applicable to other glycoproteins.

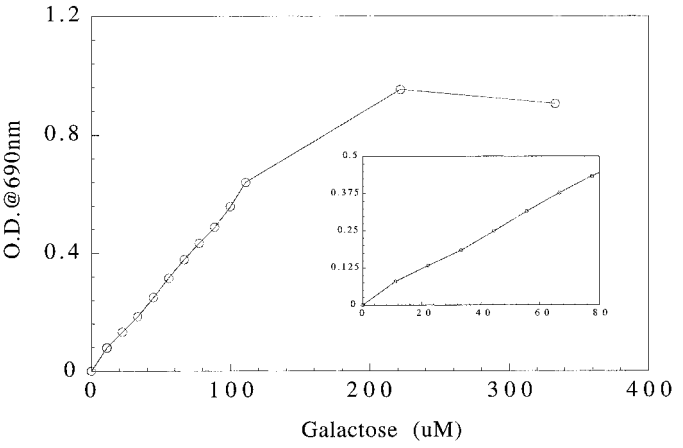


FIG. 1. Linearity of chromophore with Gal. Solution of Gal (11–333 μM) in water (150 μl) was heated with 50 μl of reagent A and 50 μl of reagent B as described in the Experimental Procedure. To the cooled reaction mixture, 250 μl of reagent C or D was added and the absorbance was measured relative to a blank of the reagent mixture without Gal, using a microplate reader. The curve was found to be linear from 11 μM to 111 μM.

EXPERIMENTAL

Materials. Diplococcus pneumoniae and bovine testes β-D-galactosidases were from Boehringer Mannheim (Indianapolis, IN). Jack bean β-D-galactosidase was from Oxford GlycoSciences (London). PNGase-F was either from Oxford GlycoSciences or from Boehringer Mannheim. Other chemicals were of analytical grade, obtained from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). NAP-5 columns were purchased from Pharmacia (Uppsala, Sweden) and used as per manufacturer's procedure. Preparation of rIgG was described previously (15).

Enzyme digestion. Samples of rIgG (1–2 mg) were loaded on NAP-5 columns and equilibrated with 100 mM sodium acetate buffer, pH 6.0. An aliquot (200–1000 μl) of the protein containing fraction was incubated with β-D-galactosidase (10–80 mU/mg protein) at 37°C for 24 hours. The reaction vial was heated in a boiling water bath for 10 min, centrifuged, and the supernatant used for colorimetric estimation.

Colorimetric assay. Gal standards (11–111 μM) and samples in 150 μl water were mixed with 50 μl of solution A (0.5 g potassium ferricyanide in 1000 ml water) and 50 μl solution B (5.3 g sodium carbonate and 0.65 g potassium cyanide in 1000 ml water). The mixture was heated in a boiling water bath for 10 min., cooled to room temperature, and 250 μl of solution C (1.5 g ferric ammonium sulfate and 1.0 g SDS in 1000 ml of 0.05 N sulfuric acid) or solution D (1.5 g ferric ammonium sulfate and 2.0 g SDS in 1000 ml of 0.2 N sulfuric acid) was added. The solution was mixed well with a pipette, transferred to a 96-well microplate and the absorbance at 690 nm measured using a microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA).

High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) of oligosaccharides. Protein samples were exchanged into 100 mM citrate phosphate buffer, pH 4.0, and adjusted to a concentration of 1 mg/mL. 40 milliUnits of β-galactosidase per mg protein was added (except where noted in Fig. 5A), and this mixture was incubated at 37°C for 18 h. Released Gal was measured on a Dionex DX-500 series instrument (Dionex Corp., Sunnyvale CA). Aliquots of the digestion mixture were injected onto a Dionex MA1 column protected by a guard column and eluted under isocratic conditions with 480 mM NaOH, 0.5 mL/min for 30 min. A Dionex Advanced Computer Interface connected to a Compaq Prolinea 4/66 computer with Dionex AI-450 (release 4.3.00) software was used to collect the data (16).

Mass spectrometry. The glycoprotein sample, before and after treatment with β-D-galactosidase, was digested with PNGase-F (*Flavobacterium meningosepticium*, Oxford GlycoSciences) in 20 mM Tris-acetate buffer, pH 8.4 for 3 h at 37°C. The released acidic and neutral oligosaccharides were analyzed separately by MALDI-TOF-MS as described elsewhere (17).

RESULTS

Assay considerations. Although several colorimetric assays are available to measure reducing sugars, the ferricyanide assay (11–14) was chosen because of its sensitivity and ease of use. UV/vis spectra of the ferricyanide assay chromophore (Prussian blue, ferric ferrocyanide) for Gal showed maximal absorbance (λ_{max}) at 690 nm. Other aldohexoses and N-acetyl hexosamines produce the same chromophore with a similar λ_{max}, but the sensitivity and linear range are different for different reducing sugars. Hence a separate standardization experiment is recommended for each reducing sugar.

Linear range for Gal. The linear range of the assay for Gal was between 11 μM to 111 μM, as shown in Fig. 1. For samples limited in amount, the assay can be scaled down to 4 μM–40 μM Gal with corresponding decreases in the amounts of reagents. Likewise, the assay can be scaled up from micromolar to millimolar amount of Gal. The lower limit is set by the ability to accurately dispense solution. The standard curve, obtained by two independent analysts on five different days, is shown in Fig. 2. The data in Fig. 2 demonstrate that the assay is reproducible and quantitative with

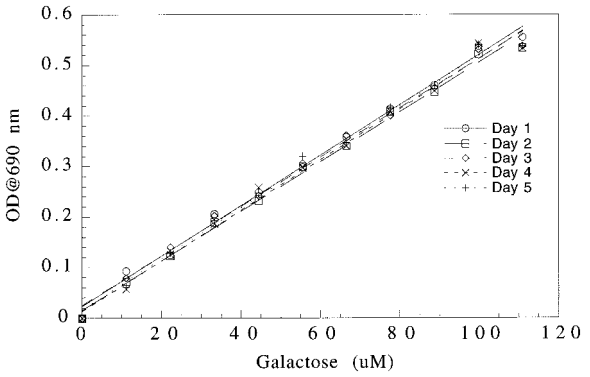


FIG. 2. Gal standard curves using the ferricyanide assay. The assay as described in Fig. 1, with 11–111 μM Gal, was performed on five different days. The experiment was repeated at least two times by two different analysts. The c.v.'s were 3–5% between analysts and days.

c.v.'s ranging between 3–5% and r^2 (square of the correlation coefficient) of 0.999 for Gal in buffer. The c.v.'s achieved for immunoglobulin samples are similar (Tables 1 and 2).

Stability of chromophore. Stability of the chromophore was determined by measuring its absorbance at various time points (Fig. 3A). The intensity decreases with time as the chromophore precipitates. The data also suggest that, with increasing amount of Gal, the rate of precipitation of the chromophore increases. By 24 h, the chromophore had entirely precipitated from all the samples. To minimize precipitation and to increase the stability of the chromophore, the sulfuric acid concentration was increased from 0.05 N to 0.2 N and the amount of SDS was increased from 1 g/L to 2 g/L. The data in Fig. 3B demonstrates that increasing the concentration of acid and SDS enhanced the stability of the chromophore over 160 min without compromising the sensitivity of the assay.

TABLE 1

Quantitation of Terminal Gal Released by β-D-Galactosidases (*D. pneumoniae*, Jack Bean, and Bovine Testes) from rIgG

	Moles of Gal released/moles of rIgG		
Enzyme	run I (n = 3)	run II (n = 3)	cv's (%)
D. pneumoniae	1.20	1.20	2
Jack Bean	0.90	0.85	3
Bovine testes	0.80	0.75	4

Note. Samples of rIgG (2 mg) were treated with β-D-galactosidase (~40 mU/mg of protein) in sodium acetate buffer (200 μl, pH 6.0) for 24 h at 37°C. The reaction was stopped by heating the mixture in a boiling water bath for 5 min., and centrifuged (3000 rpm) at room temperature for 5 min. The released Gal present in the supernatant was measured by the modified ferricyanide method. Results shown are the average of two different experiments. The coefficient of variation (c.v.) was calculated as the percentage variation from the average.

TABLE 2

Comparison of Ferricyanide Assay Results with HPAEC-PAD Results on the Quantitation of Terminal Gal Content from Various Batches of rIgG

	Moles of Gal/moles of rIgG ferricyanide assay			
Antibody samples	run I (n = 3)	run II (n = 3)	cv's (%)	HPAEC-PAD (n = 3)
Batch 1	1.2	1.2	2	1.3
Batch 2	1.5	1.6	4	1.4
Batch 3	1.8	1.9	5	1.7
Batch 4	1.4	1.4	2	1.5
Batch 5	1.5	1.4	4	1.2
Batch 6	1.2	1.3	5	1.1

Note. Different cell culture batches of rIgG samples were treated with β-D-galactosidase and the released Gal was quantitated by ferricyanide assay as described in Table 1. Alternatively, the released Gal was measured by HPAEC-PAD as described in Experimental Procedures.

Determination of terminal Gal in a recombinant chimeric monoclonal antibody (rIgG) expressed in CHO cells. Recombinant monoclonal antibodies (rIgG) expressed in CHO cells are glycosylated in the constant region of the C_{H2} domain of the heavy chain. The oligosaccharides found on rIgG are exclusively of the bi-antennary complex type with core fucosylation (18). Structures of these major oligosaccharides are shown in Scheme 1. Since parent CHO cells do not express N-acetylglucosaminyltransferase-III (the transferase responsible for adding bisecting GlcNAc to complex N-linked oligosaccharides), the recombinant monoclonal antibodies expressed in these cells do not contain oligosaccharides with bisecting GlcNAc (19). Furthermore, the majority of oligosaccharides present in recombinant antibodies expressed in CHO cells are not sialylated and the terminal Gal content of these antibodies varies from batch to batch (20). To determine the terminal Gal content of a recombinant chimeric monoclonal antibody to CD-20 expressed in CHO cells (rIgG), the molecule was treated, separately, with β-D-galactosidases from *D. pneumoniae*, jack bean, and bovine testes. The amount of released Gal was measured by the modified ferricyanide assay. The results are summarized in Table 1. It is evident that the *D. pneumoniae* enzyme was able to release a greater amount of terminal Gal from rIgG than were the enzymes from jack bean and bovine testes under the conditions used. To verify whether the *D. pneumoniae* enzyme released all of the terminal Gal residues, oligosaccharide profiling was carried out. The oligosaccharides of rIgG, before and after treatment with *D.*

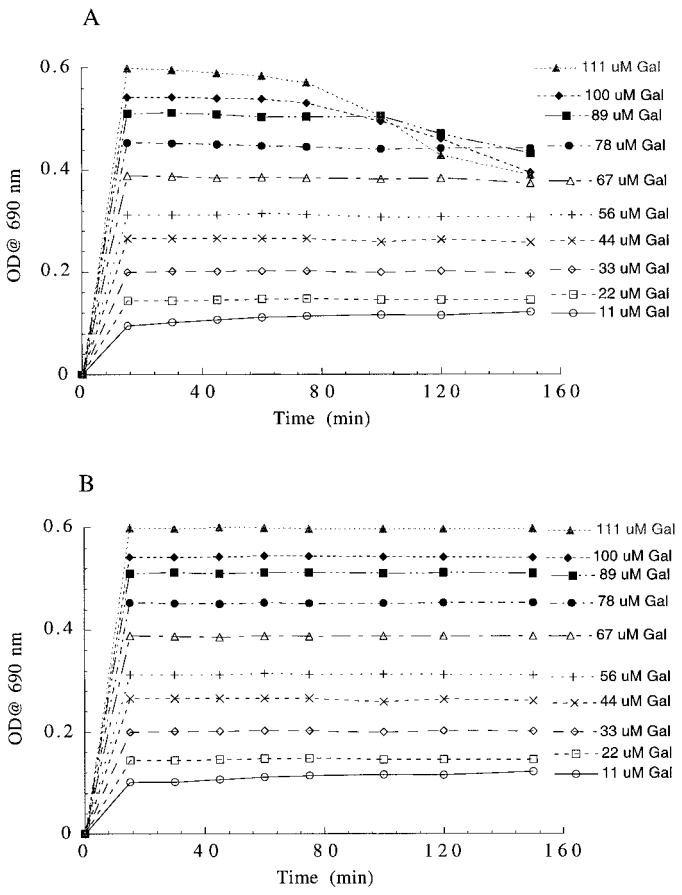
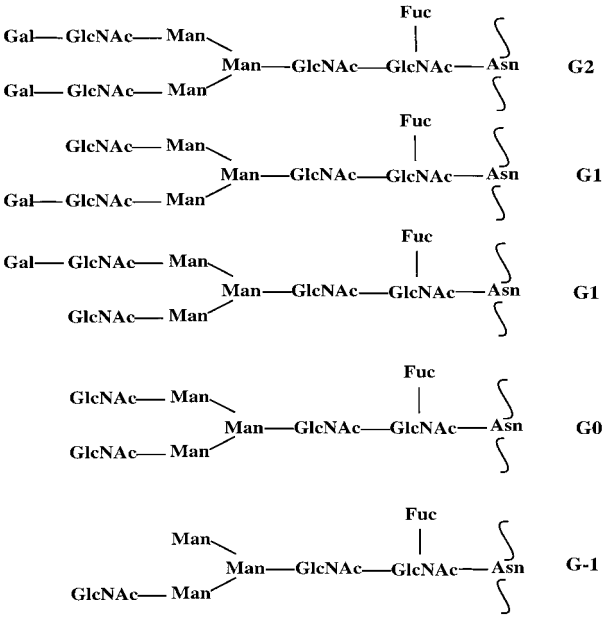


FIG. 3. Stability study of the chromophore for Gal. (A) Approximately 11–111 μM of Gal was treated as described by Park and Johnson (14) and the stability of the chromophore was measured at different time intervals using a microplate reader. (B) The experiment was repeated after increasing the concentration of sulfuric acid and SDS from 0.05 N and 0.1% to 0.2 N and 0.2% respectively.

pneumoniae enzyme, were released from the antibody by PNGase-F, which cleaves the oligosaccharides linked to asparagine, and examined by MALDI-TOF-MS (Fig. 4). The results demonstrate that the *D. pneumoniae* enzyme was able to release more than 90% of the terminal Gal residues from rIgG. Similar experiments on rIgG after treatment with either jack bean or bovine testes enzyme showed incomplete removal of terminal Gal residues. About ~30–40% of galactosylated oligosaccharides remained undigested with jack bean and bovine testes enzymes (data not shown).

The *D. pneumoniae* enzyme digestion was performed as a function of enzyme concentration, temperature, time, and pH. The results are shown in Fig. 5A–D, respectively.

Glycosylation of recombinant glycoproteins expressed in CHO cells can be affected by the cell culture conditions and hence the terminal Gal content might vary from batch to batch (20). The amount of terminal Gal residues present in six different batches of rIgG



SCHEME 1. Structure of major N-linked oligosaccharides found on monoclonal antibodies expressed in CHO cells (18). Gal = D-Galactose, GlcNAc = N-acetyl D-glucosamine, Man = D-Mannose, Fuc = L-Fucose, Asn = Asparagine.

was measured using the modified ferricyanide method. The results, summarized in Table 2, are in good agreement with the data obtained by HPAEC-PAD.

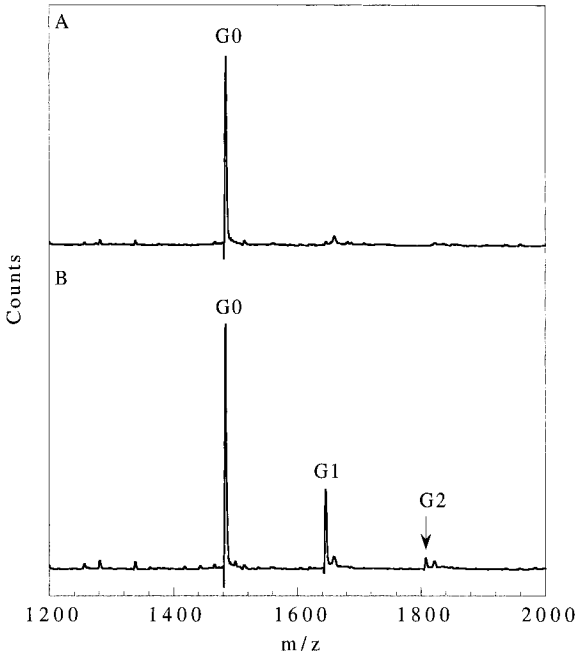


FIG. 4. MALDI-TOF-MS of rIgG oligosaccharides before and after β-D-galactosidase digestion. The rIgG (2 mg) was treated with β-D-galactosidase (*D. pneumoniae*, 40 mU/mg protein) for 24 h at 37°C. The enzyme-treated (A) and untreated (B) rIgG were purified, separately, on a Protein-A column, incubated with PNGase-F, and the released oligosaccharides were analyzed by MALDI-TOF-MS as described (17).

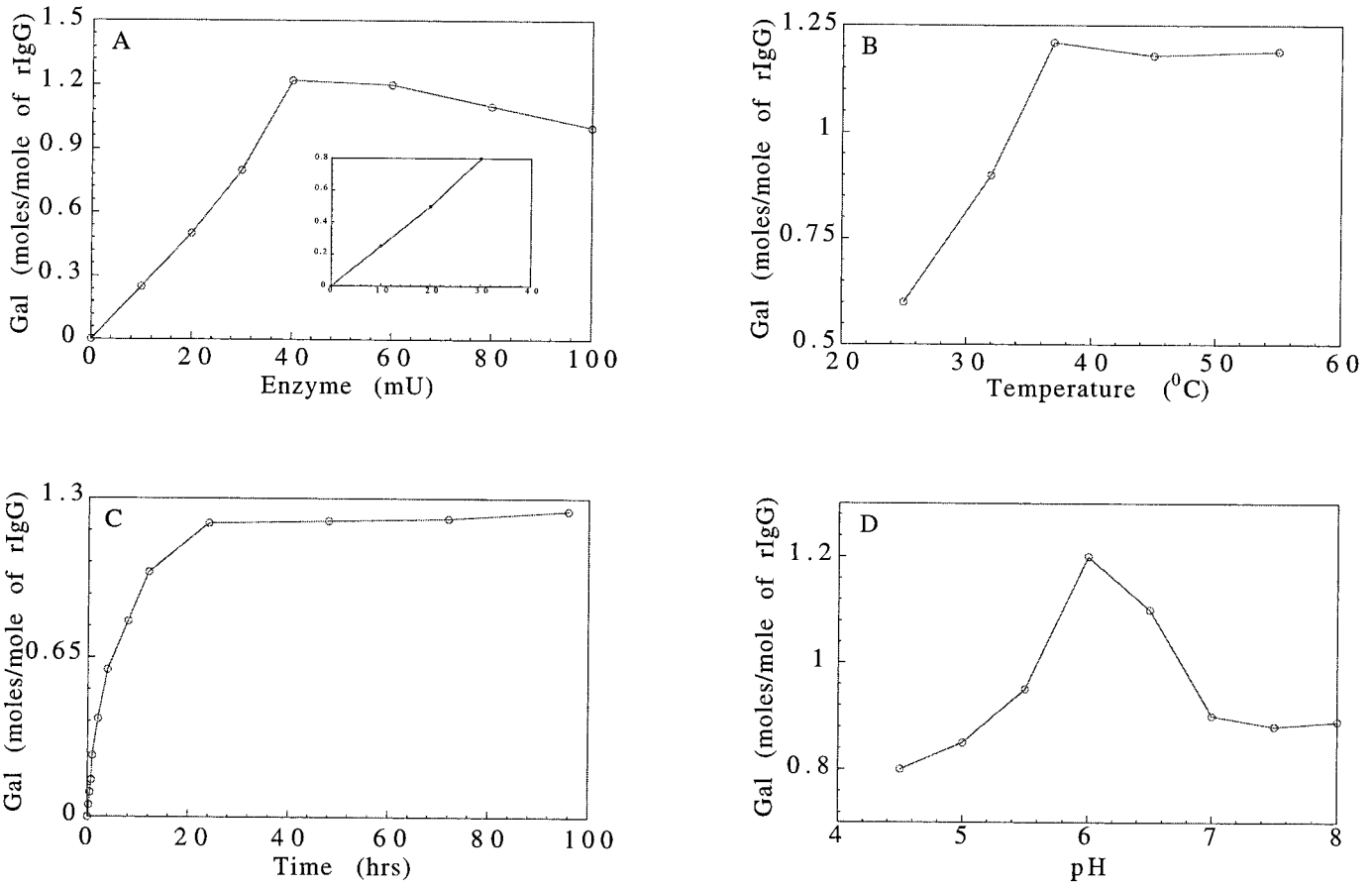


FIG. 5. Measurement of enzyme concentration (A), temperature (B), time (C) and pH (D) for β-D-galactosidase digestion of rIgG. Samples of rIgG (2 mg each) were treated with (A) β-D-galactosidase (*D. pneumoniae,* 0–100 mU/mg protein) at 37°C for 24 h in 100 mM sodium acetate buffer pH 6.0; (B) β-D-galactosidase (*D. pneumoniae,* 40 mU/mg protein) for 24 h at 25°C–55°C in 100 mM sodium acetate buffer pH 6.0; (C) β-D-galactosidase (*D. pneumoniae,* 40 mU/mg protein) at 37°C for 0-100 h in 100 mM sodium acetate buffer pH 6.0; (D) β-D-galactosidase (*D. pneumoniae,* 40 mU/mg protein) at 37°C for 24 h in 100 mM sodium acetate buffer pH 4.5–8.0 and the released Gal was quantitated by ferricyanide assay as described in Experimental Procedures.

DISCUSSION

The ferricyanide method was developed by Hagedorn and Jensen (11) to measure blood glucose levels in diabetic patients, and subsequently modified by Folin and Malmros (12, 13) and adapted to submicrodetermination by Park and Johnson (14). The method is based on the reduction of ferricyanide by reducing sugars in alkaline solution followed by formation of a chromophore (Prussian blue, ferric ferrocyanide) upon addition of ferric ions. We tried to use the Park and Johnson (14) version of the ferricyanide method to measure terminal Gal content of rIgG, but the chromophore generated with Gal was unstable and precipitated. The rate of precipitation of the chromophore increased with increasing Gal concentration. To prevent the precipitation, we increased the sulfuric acid and SDS concentration, from 0.05 N and 0.1% to 0.2 N and 0.2% respectively, which enhanced the stability and solubility of the chromophore. This modification did not alter the sensitivity and linear range of the assay, as shown in Fig. 3.

The linear range of the ferricyanide assay was determined to be between 11 μM to 111 μM. However, the sensitivity can be increased to 4 μM by scaling down the assay. Correspondingly, the sensitivity can be decreased by scaling up the assay or by dilution to quantitate millimolar amounts of Gal. In the ferricyanide assay, all reducing sugars produce the Prussian blue color. The use of a specific exoglycosidase provides the specificity required for measuring a particular terminal sugar.

To quantitate the terminal Gal residues of a recombinant immunoglobulin expressed in CHO cells (rIgG), covalently bound terminal Gal residues were released by treatment with β-D-galactosidase thus achieving selectivity for Gal. The ferricyanide assay described herein is sensitive enough to measure the Gal released from ~1–2 mg of rIgG, which is comparable to the

sensitivity of standard HPLC and HPAEC-PAD methods. The advantages of the ferricyanide colorimetric method are that it is simple and can be used in a 96-well microtiter plate format whenever high throughput is needed. Moreover, the simplicity of the method is such that no special instruments are required. A standard laboratory spectrophotometer or a microplate reader are the only instruments required for the assay along with a conventional water bath and a centrifuge.

The assay provides a convenient format for optimizing the enzyme concentration, pH, time, and temperature for the exoglycosidase digestion to release terminal Gal residues. The data presented in Fig. 5A–D indicate that the ferricyanide method can be used to measure enzyme kinetics conveniently. This method can also measure batch to batch variations in the terminal Gal content of rIgG (Table 2).

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