

Determination of the Mannose-6-Phosphate Content of a Glycoprotein

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ABSTRACT

Over 40 human diseases are caused by deficiencies in lysosomal glycoproteins known to contain mannose-6-phosphate (M-6-P). One class of these diseases is the lysosomal storage diseases where the proper enzyme does not reach the lysosome, causing a subsequent accumulation of a large molecule (e.g. glycosaminoglycan) that is not catabolized. Sometimes the cell synthesizes the required enzyme but it does not reach the lysosome because the enzyme lacks M-6-P on its oligosaccharides. The M-6-P targets the enzyme to the lysosome, and lacking M-6-P, the enzyme is instead secreted by the cell.

In order to treat these diseases, recombinant versions of the missing enzyme are produced and given to the patient intravenously or by injection, depending on the enzyme. The recombinant enzyme's attached oligosaccharides must contain M-6-P in order to cross the cell membrane and then enter the lysosome. Therefore the product must be assayed to ensure that it has its full complement of M-6-P. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) has been used after either an enzymatic or a chemical treatment to assay a variety of carbohydrates from glycoproteins, including oligosaccharides, monosaccharides, sialic acids, mono- and oligosaccharide alditols, and sugar phosphates.¹ In 2002 Zhou et al.² reported that HPAE-PAD could be used to assay the M-6-P content of a glycoprotein after acid hydrolysis.

Here we update the HPAE-PAD assay of a glycoprotein for M-6-P using a newer HPAE column for carbohydrate analysis that provides more efficient peaks, uses less eluent, and allows shorter analysis times. With more efficient peaks there is a lower probability that peaks from hydrolyzed protein will interfere with M-6-P determinations. We also update the PAD conditions for improved assay to assay reproducibility. Lacking a commercially available glycoprotein containing M-6-P, we added M-6-P to acid hydrolyzed BSA to model an assay for M-6-P. To evaluate accuracy we checked recovery versus an external standard and a second addition of M-6-P and found both were >95%. We also showed that M-6-P could be identified by treatment with alkaline phosphatase and both a subsequent assay for the disappearance of the M-6-P peak, and an assay for the resulting mannose, using a different HPAE-PAD method.

MATERIALS

Deionized water, Type 1 reagent-grade, 18.2 M Ω -cm resistivity

Alkaline phosphatase, bovine intestinal mucosa, lyophilized (Aldrich, P/N P6772-2KU)

Bovine serum albumin, essentially fatty acid free, \geq 96% (agarose gel electrophoresis), lyophilized powder (Aldrich, P/N A6003)

Mannose (C₆H₁₂O₆, Aldrich, P/N M6020)

Mannose-1-phosphate (M-1-P), sodium salt hydrate (C₆H₁₂O₉P · xNa⁺ · yH₂O, Aldrich, P/N M1755)

Mannose-6-phosphate (M-6-P), sodium salt (C₆H₁₂O₉PNa, Aldrich, P/N M3655)

Micro BCA™ Protein Assay kit (Pierce Biotechnology, P/N 23250)

Sodium acetate, HPLC grade (CH₃COONa, Aldrich, P/N 71185; Dionex, P/N 059326)

Sodium hydroxide, 50% (w/w) (NaOH, Fisher Chemicals, P/N SS254-500)

Trifluoroacetic acid [13.5 M], 1 mL ampoules (CF₃COOH, Aldrich, P/N 91701)

Tris (tris-hydroxymethylaminomethane, (NH₂C(CH₂OH)₃, Aldrich, P/N 252859)

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt P/N 72.692.005)

Filter unit, 0.2 μ m nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter for eluent preparation

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EQUIPMENT

ICS-3000 chromatography system (Dionex, Sunnyvale, CA) consisting of:

SP Single Gradient Pump module

DC Detector/Chromatography module with single or dual heating zone and 6-port injection valve

ED Electrochemical Detector equipped with cell containing disposable Au working electrode and a combination pH–Ag/AgCl reference electrode

AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray

Chromeleon® 6.8 Chromatography Workstation

Centrifuge (Eppendorf® 5400 series)

SpeedVac® evaporator

Vacuum pump (for eluent preparation)

Thermolyne® Dri-Bath heater and heating block

UV/Vis spectrophotometer

CHROMATOGRAPHY

M-6-P Determinations

Column: CarboPac® PA200 Analytical, 3 x 250 mm (P/N 062896)

Eluents*: A: 100 mM Sodium hydroxide
B: 100 mM Sodium hydroxide, 1 M sodium acetate

Method: 90% A, 10% B

Flow Rate: 0.5 mL/min

Column Temperature: 30 °C

AS Tray Temperature: 10 °C

Inj. Volume: 20 µL

Inj. Loop: 100 µL

Detection: PAD, conventional or disposable Au WE

Waveform: See Table 1.

Run Time: 30 min

Mannose Determinations (only conditions differing from above are listed)

Column: CarboPac PA20 Analytical, 3 x 250 mm (P/N 060142)

Eluents*: A: 100 mM Sodium hydroxide
B: Degassed Type 1 deionized water

Method: 10% A, 90% B from 0–12 min, 100% A, 0% B from 12–20 min,
10% A, 90% B from 20–30 min

Inj. Volume: 10 µL

* For proper preparation of eluents for HPAE-PAD, please see Dionex Technical Note 71.³

Table 1: Waveform A, Four-Potential Carbohydrate Waveform⁴

Time (sec)	Potential (V) (Ag/AgCl reference)	Gain*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

* These parameters are not used on older model Dionex chromatography systems.

PREPARATION OF 1 mM M-1-P AND M-6-P STOCK STANDARDS

6.0 mg of sodium M-1-P hydrate (FW 300.15) and 5.8 mg of sodium M-6-P (FW 282.12) were each dissolved in 20.0 g deionized water (two solutions) and stored at –20 °C.

PREPARATION OF M-1-P AND M-6-P WORKING STANDARDS

0.5, 1, 2, 5, 10, 20, and 50 µM working standards were prepared by diluting 5, 10, 20, 50, 100, 200, and 500 µL of each sugar phosphate into separate 20 mL scintillation vials with deionized water to 10.0 g total weight. A 100 µM M-6-P spiking standard solution was prepared by diluting 500 µL of the 1.0 mM M-6-P stock standard to 5.0 g total weight. The working standards were stored at 4 °C.

PREPARATION OF MANNOSE STOCK AND WORKING STANDARDS

Dissolve 36.0 mg of mannose in 20.0 g of Type 1 deionized water. To prepare 1, 5, 10, 20, and 50 µM mannose working standards from 10.0 mM mannose, dilute 1, 5, 10, 20, and 50 µL in separate 20 mL scintillation vials with deionized water to 10.0 g total weight.

PREPARATION OF 50 mM TRIS BUFFER (pH 9) AND ALKALINE PHOSPHATASE WORKING SOLUTIONS

To dephosphorylate M-1-P and M-6-P containing samples with alkaline phosphatase prepare 50 mM Tris buffer (pH 9) and 2 units/µL alkaline phosphatase according to the instructions in references 2 and 5.

SAMPLE PREPARATION

Bovine Serum Albumin (BSA) Stock Solution

Dissolve 25 mg of BSA solid with 5 mL of Type 1 deionized water in a 20 mL HDPE scintillation vial and store at -20 °C.

Acid Hydrolysis

BSA samples were hydrolyzed with 6.75 M trifluoroacetic acid (TFA) in the Thermolyne Dri-Bath at 100 °C for 1.5 h.² The BSA samples were prepared by pipetting 20 µL of 5 mg/mL BSA into 1.5 mL Sarstedt screw top tubes followed by addition of 125 µL of concentrated TFA (13.5 M) and 125 µL of deionized water. The blanks were prepared similarly by substituting 20 µL deionized water for the sample. After hydrolysis, the vials were quenched in ice water, microfuged for 2 min, and dried using a SpeedVac evaporator at room temperature and vacuum (~0.5–1 Torr). The dried samples and blanks were stored at -40 °C, thawed at room temperature, re-constituted with 200 µL of deionized water, and centrifuged for 5 min prior to use. Reconstituted TFA-hydrolyzed BSA samples were spiked with 2, 3, 5, or 10 µM M-6-P by pipetting 4, 6, 10 or 20 µL of 100 µM M-6-P, respectively.

Alkaline Phosphatase Treatment

To prepare samples for mannose and M-6-P determinations after dephosphorylation, pipette 50 µL of 50 mM Tris buffer (pH 9) and 3 µL of 2 units/µL alkaline phosphatase into the 1.5 mL screw top vials containing one of the replicates of the re-constituted acid-hydrolyzed samples described above. Prepare separate 10 µM M-6-P and M-1-P control samples in the same way by using 197 µL deionized water, 50 µL of 50 mM Tris buffer (pH 9), 25 µL of 100 µM M-6-P or 100 µM M-1-P, and 3 µL of 2 units/µL alkaline phosphatase. Prepare 3 µM M-6-P or M-1-P control samples using 7.5 µL of 100 µM M-6-P or 100 µM M-1-P rather than 25 µL. Digest all samples at 37 °C for 5 h. Quench the vials in ice, centrifuge at 14,000 rpm for 5 min, and dry by SpeedVac evaporator at room temperature and vacuum (~0.5–1 Torr) for 2–3 h. Store the dried solutions at -20 °C. Reconstitute the samples with 200 µL deionized water prior to analysis.

Heat-Quenched Dephosphorylation Samples

Dephosphorylated samples were spiked with M-6-P as a control. To prepare these samples, the reconstituted dephosphorylated samples and controls were heat-quenched in boiling water for 5 min. Then 5 µL of 100 µM M-6-P was added to 100 µL of sample.

Determination of Protein Concentration

The Micro-BCA test kit was used to measure protein concentrations.

RESULTS AND DISCUSSION

Figure 1 shows the separation of M-1-P and M-6-P on the CarboPac PA200 column. The PA200 has similar selectivity to older CarboPac columns (e.g. PA100) but has a smaller particle size to allow more efficient separations while using less eluent and thereby generating less waste. Additionally, a lower sodium acetate concentration is needed for elution. A CarboPac PA100 based assay that separated M-1-P and M-6-P was used to evaluate the activity of phosphomannosemutase.⁶

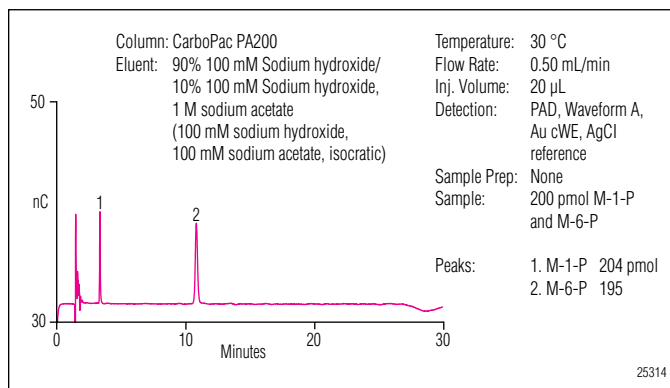


Figure 1. Separation of M-1-P and M-6-P.

Due to the lack a commercially available protein known to contain M-6-P, we used the acid hydrolysis conditions of reference 2 to hydrolyze BSA to create a “generic” acid hydrolyzed protein matrix to which we then added M-6-P. Figure 2 shows the method blank and the same blank with the addition of 200 pmol M-6-P. The blank shows no peaks that will interfere with a M-6-P assay and that M-6-P is fully recovered (226 pmol measured with the calibration curve).

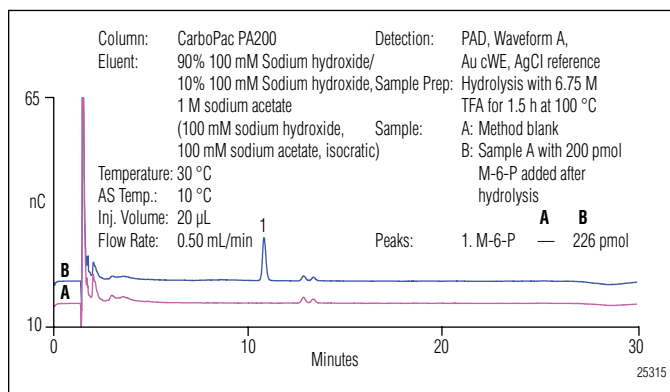


Figure 2. A comparison of A) TFA-hydrolysis blank to B) blank spiked with 200 pmol of M-6-P after hydrolysis. Chromatography conditions same as Figure 1.

M-6-P was also added to the acid-hydrolyzed BSA. Figure 3 shows the separation of the acid hydrolyzed BSA and the sample with additions of 200 and 400 pmol M-6-P. The acid-hydrolyzed BSA has a number of early eluting peaks, but using our separation conditions, nearly all elute before M-6-P and do not interfere with its determination. If there was interference, lowering the acetate concentration, which would lengthen the separation time, might resolve the M-6-P from the interference. The two additions of M-6-P to the matrix, with good recovery for both, show that PA200 column has enough capacity under these conditions to bind the M-6-P in the presence of 130 pmol acid-hydrolyzed BSA.

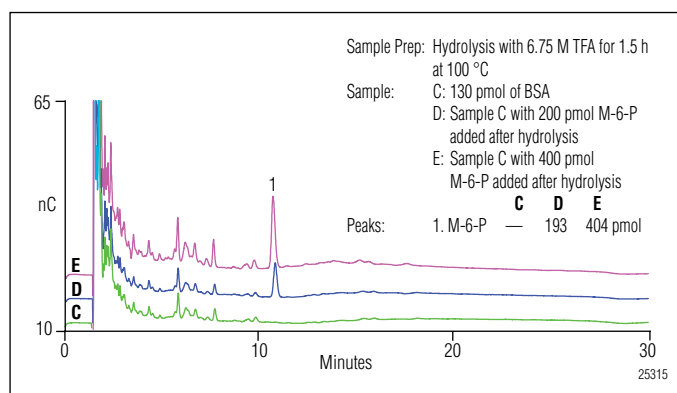


Figure 3. Comparison of BSA after TFA hydrolysis to replicate samples spiked with M-6-P. Chromatography conditions same as Figure 1.

If one assays a protein for M-6-P and observes a peak with the retention time of M-6-P, one should still confirm that the peak is M-6-P and not an electroactive piece of the protein or other component of the protein sample. Treatment with alkaline phosphatase (AP) and subsequent HPAE-PAD assay of the sample for disappearance of the putative M-6-P is an easy way to confirm the presence of M-6-P. Figure 4 shows the chromatography of acid-hydrolyzed BSA that had been spiked with M-6-P and then treated with AP. Trace B shows a M-6-P standard that was treated with AP to serve as a positive control. Note the absence of the M-6-P in both traces.

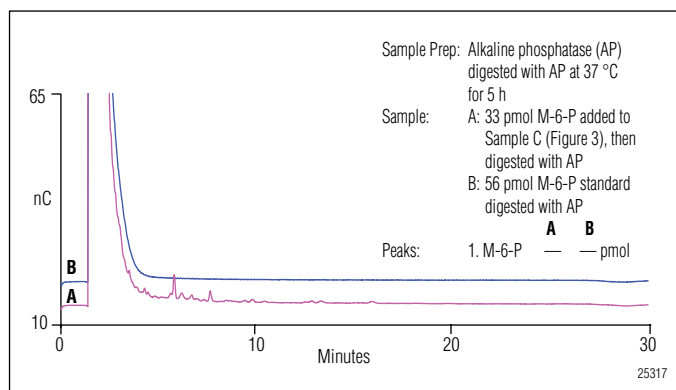


Figure 4. The absence of M-6-P after alkaline phosphate digest. Chromatography conditions same as Figure 1.

The result in Figure 4 could be due to an overload of the column with the AP buffer. To test if the result of Figure 4, dephosphorylation of M-6-P, is valid, we performed two tests. In the first test we heat-quenched the AP digests to denature AP, and then spiked the samples previously digested with AP with M-6-P. This experiment is shown in Figure 5 and shows the presence of M-6-P in both the standard and the acid-hydrolyzed BSA sample, and therefore that the AP digest does not overload the column.

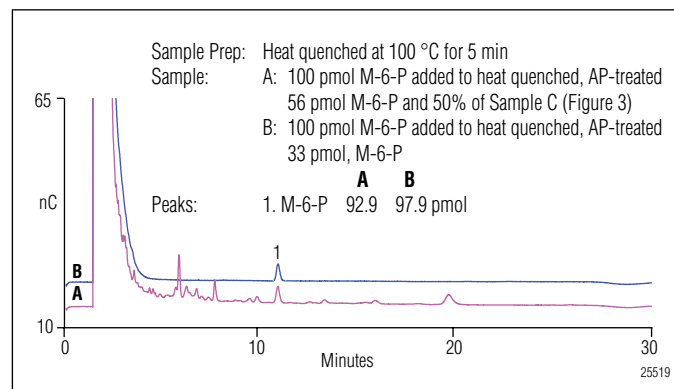


Figure 5. 100 pmoles of M-6-P spiked into heat quenched alkaline phosphatase-treated samples. Chromatography conditions same as Figure 1.

The second test is an HPAE-PAD assay for mannose. If the AP removed phosphate, we should be able to find free mannose in the sample. Figure 6 shows that the samples do contain the expected amount of free mannose.

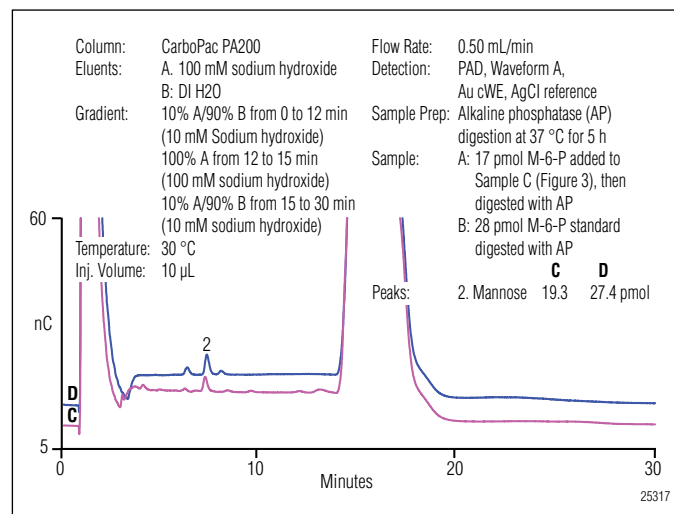


Figure 6. Assay of mannose in alkaline phosphatase-treated samples.

4 Determination of the Mannose-6-Phosphate Content of a Glycoprotein

Quantitative results for the experiments in Figures 2 through 6 are shown in Table 2.

CONCLUSIONS

1. HPAE-PAD can accurately assay M-6-P in acid hydrolyzed protein without sample derivatization or other pretreatment.
2. The presence of M-6-P can be confirmed by alkaline phosphatase treatment and assay for either mannose or the absence of M-6-P.

REFERENCES

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2. Q. Zhou J. Lyazike T. Edmunds and E. Higgins, *Anal. Biochem.* **2002**, *306*, 163.
3. Dionex Technical Note 71.
4. Dionex Technical Note 21.
5. Dionex Application Note 202.
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Table 2: M-6-P and Mannose Assay Results

Sample	M-6-P Added (pmole)	M-6-P Measured (pmole)	Recovery (%)	M-6-P in Sample (pmole)	Mannose Measured (pmole)	Recovery (%)
TFA Blank	226	226 ± 8	100	—	—	—
TFA-Hydrolyzed BSA #1	204	193 ± 2	94.6	—	—	—
TFA-Hydrolyzed BSA #2	406	404 ± 1	99.5	—	—	—
TFA-Hydrolyzed BSA #1 Treated with AP	—	ND	—	11	10.5 ± 0.8	95.5
TFA-Hydrolyzed BSA #2 Treated with AP	—	ND	—	22	20.7 ± 1.7	94.1
M-6-P Treated with AP	—	ND	—	28	27.4 ± 0.8	97.9
M-6-P Treated with AP Heat Quenched	102	97.9 ± 0.5	96.0	—	—	—
TFA-Hydrolyzed BSA #1 Treated with AP Heat Quenched	102	92.9 ± 0.5	91.1	—	—	—

ND – not detected

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