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Short communication

The influence of sodium hyaluronate molecular weight on protein content according to Lowry and Coomassie blue assays

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ABSTRACT

Sodium hyaluronate (HA) of various molecular weights (MW) were prepared using acid hydrolysis, heat degradation, ultrasonic degradation, and irradiation on the same production batch. The molecular weights were determined using size exclusion chromatography (SEC) with in-line multi-angle laser light scattering (MALLS) and refractive index (RI) detection. The protein content for these samples were determined with the European Pharmacopoeia method for protein content, the Lowry assay and the Coomassie blue (BioRad, Bradford) assay. According to the Lowry assay, the determined protein contents increased with decreasing MW for all series of degraded HA. In the Coomassie blue assay, the protein level was independent of the MW. This was also shown by analysing a series of commercially available low molecular weight (LMW) hyaluronic acid products with the two assays.
Further, degraded HA that had been reduced with $NaBH_4$ were studied, and also these gave higher protein contents in the Lowry assay, showing that it is not the increase in reducing ends that gives this artificial high responds.
In general, the Coomassie blue assay was shown to be a better method for determination of protein contents in HA.

1. Introduction

There are several assays available for determination of total amount of protein in a sample, and there are several reviews where these methods are compared (Dawnay, Hirst, Perry, & Chambers, 1991; Jenzano, Hogan, Noyes, Featherstone, & Lundblad, 1986; Peterson, 1979; Peterson, 1983; Sapan, Lundblad, & Price, 1999). The Lowry (also called enhanced copper), BCA (Smith, bicinchoninic acid), and Coomassie blue (Bradford, BioRad) are some of the most common methods but other assays are also under development, such as the Ninhydrin assay (Sapan et al., 1999; Mikkelsen & Corton, 2004). The protein assays are most often based on a reaction between different amino residues of the proteins such as tyrosine, tryptophan, and cysteine for the Lowry assay; arginine, tyrosine, phenylalanine, and tryptophan for the Coomassie blue assay (Sapan et al., 1999; Mikkelsen & Corton, 2004). However, many of these assays are also sensitive to the presence of other components such as nucleic acids, lipids, sugars, and other miscellaneous substances present in the sample. Moreover, some assays have a limited linear range, low accuracy, low reproducibility, etc. (Sapan et al., 1999). Additionally, bovine serum albumin (BSA) which is the most commonly used reference compound, is not always representative for the proteins present in the analysed sample and is not always suitable for the specific assay.

Due to the influence of the interferences present in the sample some protein assays provide overestimated values or are somehow influenced by other species in the sample thus providing erroneous values. For instance, it is well known that the presence of polysaccharides, containing free aldehyde or keto groups, can interfere with some of the assays. An example is the BCA method that is widely used for both protein and polysaccharide determination (Gupta et al., 1997; Milton & Mullen, 1992; Mopper & Melvin Gindler, 1973). Thus, for samples where the protein content is going to be determined and contains polysaccharides (or vice versa), the BCA method is not very suitable. Due to this reason it is important to have a method that is specific and is affected as little as possible by the presence of other substances in the sample.

During this study the effect of the molecular weight of sodium hyaluronate (HA) and its influence on different protein assays have been investigated. As HA is a polysaccharide, it is therefore of importance to have a method that is specific for proteins and is not affected by the presence of polysaccharides. The effect of the molecular weight of HA on the protein determination using two assays, Lowry and Coomassie blue, have been studied more in details. HA is a linear, negatively charged polysaccharide composed of alternating repeats of β-(1→3) bound *N*-acetyl D-glucosamine (GlcNAc) and β-(1→4) bound D-glucuronic acid (GlcA). HA is a natural

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component of the extracellular matrix of all mammalian tissues. Commercially, it is available as an extract from rooster combs, produced by fermentation of *Streptococcus* sp. (class 2 micro-organisms), and also lately through biosynthesis from *Bacillus subtilis*. Sodium hyaluronate has found several applications in cosmetics, wound healing, ophthalmology, orthopaedics, and drug delivery due to its biodegradability and biocompatibility. One of its unique properties is the high water-binding capacity and the interesting visco-elastic behavior.

2. Experimental

Lowry Assay (prepared according to the European pharmacopoeia (Ph. Eur.) (http://online.edqm.eu, 2007); a cupri-tartaric solution was prepared by dissolving 10 g/l of copper sulphate and 20 g/l of sodium tartrate in 0.2 M sodium hydroxide in Milli-Q water. To 1.0 ml of the solution, 50 ml of 40 g/l anhydrous in 0.2 M sodium hydroxide solution was added. A phosphomolybdotungstic reagent was prepared by dissolving 100 g of sodium tungstate ($Na_2WO_4{\cdot}2H_2O$) and 25 g of sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$) in 700 ml of Milli-Q-water. Hydrochloric acid (100 ml) and of phosphoric acid (50 ml) were added whereby the mixture was heated and refluxed in glass apparatus for 10 h. Lithium sulphate (150 g), Milli-Q-water (50 ml) and a few drops of bromine were added and boiled in order to remove the excess of bromine (15 min). After that the solution was allowed to cool, diluted to 1000 ml with Milli-Q-water and filtered.

A stock solution of BSA (96%) at 0.5 mg/ml in Milli-Q-water was used to prepare the standards. Five dilutions of the stock solution containing between 1.25 and 50 μg/ml of were prepared as standards.

Freshly prepared cupri-tartaric solution (1.0 ml) was added to a test tube whereby 1.0 ml of Milli-Q water (blank) or 1 g of test solution (approximately 10 mg of HA per ml) was added. The solution was mixed and after proximately 10 min, 0.20 ml of a mixture of equal volumes of Milli-Q-water and phosphomolybdotungstic reagent were added whereby the solution was mixed and very shortly ultrasonicated in order to remove air bobbles. After 20 min the absorbance was measured at 750 nm and compared with the calibration curve.

Coomassie blue assay (prepared according to the Ph. Eur. (http://online.edqm.eu, 2007)); the same solution of 10 mg/ml HA in Milli-Q as prepared for the Lowry assay was used during the measurements. A standard curve of BSA in water was prepared as above in the concentration range of 1.25–20 μg/ml. HA (800 μL) was mixed with a 200 μL Coomassie blue reagent and the solution was mixed and incubated for 30 min. The absorbance was measured at 595 nm and compared with the calibration curve.

Reduction of the reducing ends of the HA was carried out by dissolving HA of various molecular weights in Milli-Q water at 1% (w/w) concentration. Sodium borohydride was added to an approximate concentration of 1% (w/w) whereby the solution was stirred overnight. Acetic acid was added in order to control the reaction conditions and to deactivate the access of sodium borohydride. After the reduction, the samples were transferred to a dialysis tube of regenerated cellulose (Spectra Por, Breda, The Netherlands) with a MW cut-off of 3 kDa. The outer solution was changed until it had the same conductivity as Milli-Q water. The dialysed sample was then transferred to a beaker, frozen, and lyophilized.

Molecular weights were determined by using size exclusion chromatography with online multi-angle laser light and refractive index detection (SEC-MALLS-RI, Wyatt Technology Corp., Santa Barbara, CA); The HA (approximately 0.3 g/l HA in buffer) samples were analysed using SEC-MALLS-RI (mobile phase: 150 mM NaCl, 50 mM NaH_2PO_4, pH 7.0, 0.5 ml/min, injected volume: 0.5 ml). The columns used were PL aquagel OH-40/OH-50/OH60 with a Waters Alliance HPLC system Waters 2410 RI detector and Wyatt MALLS detector. The data were processed using the ASTRA 4.90.07 software from Wyatt Technology Corp. Preparation of the different molecular weights by acid hydrolysis is described elsewhere (Tømmeraas & Melander, 2008). Samples degraded by irradiation, ultrasound, and heat treatment were supplied by Novozymes Biopolymers A/S (Bagsvaerd, Denmark).

All chemicals were of P.A. grade unless other mentioned. The HA was supplied by Novozymes Biopolymer A/S.

HA of different molecular weights can be prepared using different degradation methods. In this study, the native HA was degraded using four different methods; beta irradiation, ultrasonication, acid hydrolysis and heat treatment. Beta irradiation degradation was performed by uniformly exposing the HA to 5–30 kGy. Ultra-sonication degradation was performed by preparing a 0.5% (w/w) HA solution and re-circulate the solution with a peristaltic pump over a Branson (B-30) ultrasonicator. Samples were collected at different intervals. Heat treatment degradation was performed by incubating HA at 105 °C for different amount of time. Acid hydrolysis was performed by hydrolysing the HA in aqueous 0.5 M HCl at 60 °C and collecting samples at different intervals: 0.60, 1.25, 2.83, 6.48, and 18.0 h. Immediately after collection the samples were cooled and neutralized with equimolar amount of NaOH (4 M NaOH). ^{1}H NMR spectroscopy have show that no de-*N*-acetylation or other side reactions occurred during the hydrolysis of HA. Degradation of HA by acid hydrolysis has been described by Tømmeraas and Melander (2008).

3. Results and discussion

In order to evaluate the influence of different degradation methods on protein content according to the Lowry assay, HA have treated with different known degradation methods. As it can be seen in Fig. 1. HA degradation to lower molecular weights have a strong influence on the Lowry assay. Additionally, the different degradation methods seem to affect the Lowry assay to different extends: acid hydrolysis affects the Lowry assay the least, followed by ultrasound, and finally irradiation and heat degradation. The general trend is that protein content in HA according to the Lowry assay increases as the molecular weight of the HA decreases. At the higher molecular weight of the HA starting material (750 kDa), the protein content seems to level off around 0.05–0.1%. The relative standard deviation of the protein determination for the Lowry assay during the study was below 10% (data not shown).

In order to see if there were any differences between the Lowry and the Coomassie blue assays, five HA samples of various MW were prepared by acid hydrolysis whereby the protein content were determined using the two assays. The results are shown in Fig. 2. This evaluation confirmed that the values obtained using Lowry assay were influenced by the MW while the Coomassie blue assay gave constant stable values independently of the MW of HA. This is expected since it is the same sample that has been hydrolysed over time to different molecular weights.

It may be argued that the Coomassie blue does not give the same response as the Lowry assay to the presence of proteins in a sample. It has been shown previously that the relative response for BSA standards is not the same for Coomassie blue and Lowry assays. We therefore measured protein content in HA of constant molecular weight using the two assays in a positive and negative control. Results showed similar values in the controls independently of assay. Thus, there are no direct indications that the results obtained are due to the differences in sensitivity for proteins for the two methods in presence of HA. However, it should be noted that it has been reported in several articles that the Lowry assay gives higher response to proteins compared to the Coomassie

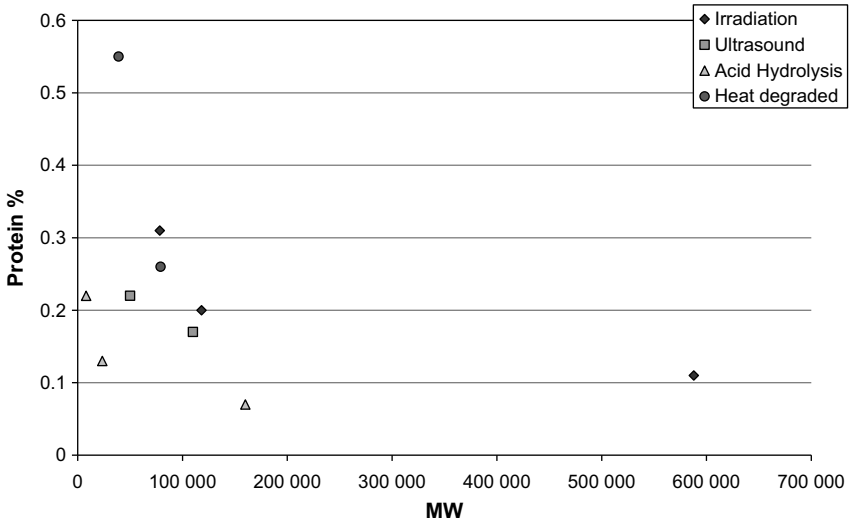

Fig. 1. Protein content according to the Lowry assay versus HA molecular weight (MW). The protein dependence on the MW of HA degraded using different methods is shown.

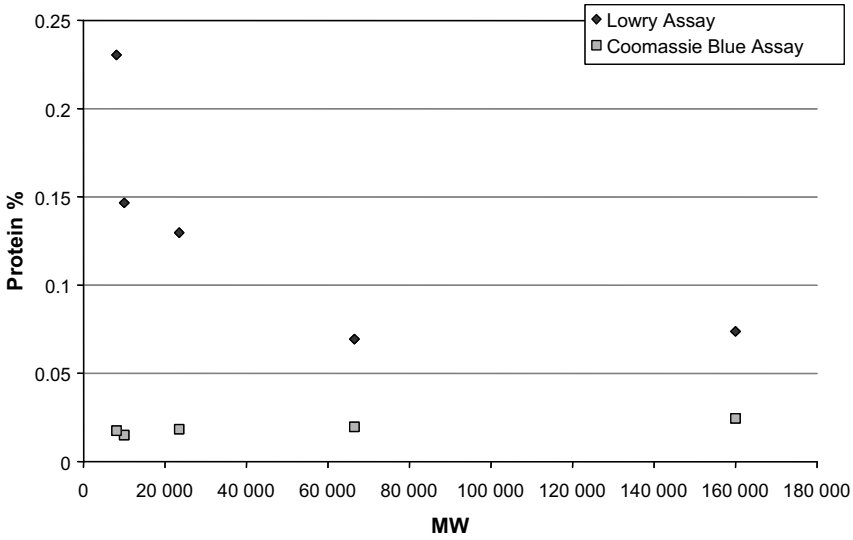

Fig. 2. Comparison of Lowry and Coomassie blue assays of HA of different MW prepared by acid hydrolysis (0.5 M HCl, 60 °C). The MW was measured to 160, 66.5, 23.5, 10, and 8 kDa by SEC-MALLS-RI. Protein content of intact starting material was measured to 0.06%.

blue assay (Keller & Neville, 1986; Kirazov, Venkov, & Kirazov, 1993; Lane, Federman, Flora, & Beck, 1986; Slater & Trybul, 1994).

As a complement to the different molecular weights HA produced from the same starting material, four different commercial low molecular weight and one high molecular weight HA samples from different manufacturers were investigated for protein content using the two assays. The results summarized in Table 1 clearly show a significant difference between the Coomassie blue and the Lowry assay with all low molecular weight samples having higher protein content according to the Lowry assay. However, only a very little difference can be seen between the two assays for the high molecular weight sample. Thus, there is no indication that the increase in the Lowry assay for low molecular weight HA is due to the presence of a protein contaminant. If that would be the case, one could expect that the Coomassie blue assay also would give higher values after degradation to low molecular weight HA. We could conclude from this study that there is a significant trend that all low molecular weight HA have higher protein contents according to Lowry assay compared to the Coomassie blue assay.

Table 1
Protein content according to Lowry and Coomassie blue assay for different low molecular weight (LMW) and one high molecular weight (HMW) HA samples

	Lowry % protein	Coomassie % protein	Molecular weight (Da)
LMW manufacturer 1	0.27	0.06	48,000
LMW manufacturer 2	0.59	0.04	34,000
LMW manufacturer 3	0.10	0.02	40,000
LMW manufacturer 4	0.50	0.02	35,000
HMW manufacturer 1	0.07	0.04	750,000

We have conducted additional experiments in order to better understand the differences between Lowry and Coomassie blue assay and eventually to find a possible explanation. As known from literature, some protein assays are sensitive to the presence of sugars, thereby giving false positives to the assays. This is most often due to the presence of the reducing end of the sugar chains (Mopper & Melvin Gindler, 1973). We believe that HA samples of high molecular weight do not contain any high amount of reducing ends

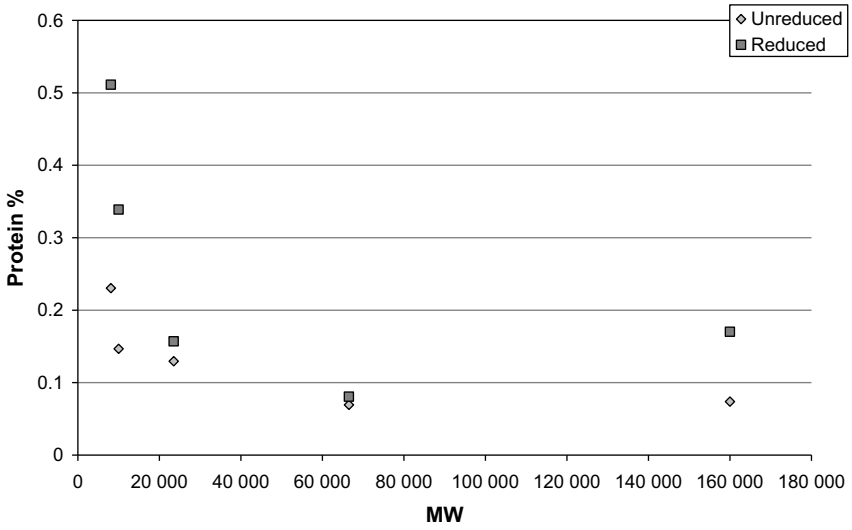

Fig. 3. Protein content using Lowry assay of sodium hyaluronate of various molecular weights (prepared by acid hydrolysis), before and after reduction with $NaBH_4$.

and thereby do not affect the Lowry assay to a significant extent. However, as it has been shown in Fig. 1, when the MW decreases, the number of reducing ends increase and their effect on the Lowry assay increases. Since the degradation of HA using acid hydrolysis has in literature shown to be completely random (Tømmeraas & Melander, 2008), the increase of the reducing ends during acid degradation is expected to be similar. This same trend can be seen in Figs. 1 and 2. As a consequence of the increase, an attempt was made to remove the reducing ends of the HA followed by a new determination of the protein content using the Lowry assay. Reduction of the reducing end (aldehyde) of HA to glycitol was achieved by adding a surplus of the reduction agent $NaBH_4$, followed by complete removal of the resulting borate salts by dialysis. In order to investigate if there was any relationship between the presence of reducing end and the protein content obtained using Lowry assay, different molecular weights of HA obtained from the same starting material were evaluated.

As it can be seen in Fig. 3, there is no clear difference in the protein content according to the Lowry assay for the reduced and unreduced samples when the MW exceeds 20 kDa. However, for the very low MW samples where the MW is below 10 kDa, the unreduced samples have lower protein content according to the Lowry assay. There is no possible explanation to this finding. The samples were investigated using ^1H NMR spectroscopy and no signals from reducing ends were seen in the anomer region, confirming complete reduction of the reduced samples (results not shown). Additionally, the MW of the reduced samples were determined and found unchanged after the reduction. Hence, no explanation can be given to this phenomenon at this time.

4. Conclusions

In this study, it has been shown that there is a strong artificial protein response in the Lowry assay when analysing low molecular weight sodium hyaluronate samples. The lower the molecular weight is, the higher the influence on protein content using the Lowry assay. We could conclude from the study that the Lowry assay is not suitable for protein determination in sodium hyaluronate of lower molecular weight. Instead, the Coomassie blue (Bradford, BioRad) assay has been investigated and shown to be a more suitable total protein assay since it gives stable response, independently on the molecular weight of sodium hyaluronate.

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References

Dawnay, A. B. S. J., Hirst, A. D., Perry, D. E., & Chambers, R. E. (1991). A critical-assessment of current analytical methods for the routine assay of serum total protein and recommendations for their improvement. *Annals of Clinical Biochemistry, 28*, 556–567.

Gupta, R. K., Chang, A. C., Griffin, P., Rivera, R., Guo, Y. Y., & Siber, G. R. (1997). Determination of protein loading in biodegradable polymer microspheres containing tetanus toxoid. *Vaccine, 15*(6–7), 672–678.

http://online.edqm.eu. European Pharmacopoeia 5.8.

Jenzano, J. W., Hogan, S. L., Noyes, C. M., Featherstone, G. L., & Lundblad, R. L. (1986). Comparison of 5 techniques for the determination of protein-content in mixed human-saliva. *Analytical Biochemistry, 159*(2), 370–376.

Keller, R. P., & Neville, M. C. (1986). Determination of total protein in human milk – Comparison of methods. *Clinical Chemistry, 32*(1), 120–123.

Kirazov, L. P., Venkov, L. G., & Kirazov, E. P. (1993). Comparison of the Lowry and the Bradford protein assays as applied for protein estimation of membrane containing fractions. *Analytical Biochemistry, 208*(1), 44–48.

Lane, R. D., Federman, D., Flora, J. L., & Beck, B. L. (1986). Computer-assisted determination of protein concentrations from dye-binding and bicinchoninic acid protein assays performed in microtiter plates. *Journal of Immunological Methods, 92*(2), 261–270.

Mikkelsen, S. R., & Corton, E. (2004). *Bioanalytical Chemistry*. Toronto: Wiley-Blackwell.

Milton, J. D., & Mullen, P. J. (1992). The effect of reducing and nonreducing sugars on the bicinchoninic acid reaction for protein determination. *Clinica Chimica Acta, 208*(1–2), 141–143.

Mopper, K., & Melvin Gindler, E. (1973). A new noncorrosive dye reagent for automatic sugar chromatography. *Analytical Biochemistry, 56*(2), 440–442.

Peterson, G. L. (1979). Review of the Foline phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Analytical Biochemistry, 100*(2), 201–220.

Peterson, G. L. (1983). Determination of total protein. *Methods in Enzymology, 91*, 95–119.

Sapan, C. V., Lundblad, R. L., & Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry, 29*, 99–108.

Slater, J. E., & Trybul, D. E. (1994). Immunodetection of latex antigens. *The Journal of Allergy and Clinical Immunology, 93*(5), 825–830.

Tømmeraas, K., & Melander, C. (2008). Kinetics of hyaluronan hydrolysis in acidic solution at various pH values. Biomacromolecules, accepted for publication.