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ARTICLE TYPE

An Improved Assay for the Spectrophotometric Determination of Chondroitinase ABC Activity

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Chondroitinase ABC (chABC) has evolved as a promising therapeutic for the degradation of inhibitory chondroitin sulfate proteoglycans (CSPGs) after injury to the central nervous system (CNS). The enzymatic breakdown of CSPGs by administered chABC reinstated plasticity as well as axon regeneration; leading to improved CNS recovery. Thus in an effort to develop and evaluate treatments of CNS injury utilising chABC the accurate detection of levels of CSPGs and more importantly activity of chABC activity is of paramount importance. Here we present a spectrophotometric assay suitable for accurately and quantitatively measuring chABC activity, based on the use of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1). This novel WST-1 based assay was compared to two other assays: measurement of alkene generation at 232 nm and a 1,9 dimethyl methylene blue (DMMB) assay which have been used previously for the detection of chABC activity and CSPGs respectively. Comparison of these assays demonstrated that the WST-1 assay was quantitative, efficient, cheap and easy to perform and overall was superior in the detection of chABC activity at all concentrations when compared to the other methods tested.

Introduction

Chondroitin sulfate proteoglycans (CSPGs) are a diverse group of extracellular compounds consisting of a core protein and one or more covalently attached sulfated glycosaminoglycan (GAG) side chains.¹ CSPGs play critical roles in a range of biological processes and diseases, providing a cellular framework to direct and at times inhibit cell growth and tissue formation within the extracellular matrix *via* activation of cell signalling pathways.^{2,3} Within the central nervous system (CNS) CSPGs play an important role in axon guidance during development as well as in the formation and maturation of perineuronal nets (PNNs).³ Formation of PNNs has been well characterised to signal the maturation of synaptic connections and in turn the closing of the CNS's plastic phase.³⁻⁵ Following injury to the CNS a range of biochemical changes occur, including a sharp rise in the levels of

CSPGs associated with the formation of the glial scar at the primary injury.⁴ It has been shown that the presence of CSPGs at the site of injury results in an environment that is inhibitory to axon regeneration and axonal plasticity, two important factors required for restoration of function post-CNS injury.^{1,4} Chondroitinase ABC (chABC), has been used to successfully degrade CSPGs and PNN structures at the site of a CNS injury both *in vitro* and *in vivo*, which in turn resulted in axon regeneration and more importantly the reintroduction of plasticity at the injury site.^{1,4,6} Despite this proof-of-concept demonstration of the use of chABC in treating CNS injury, several limitations have been reported.

Figure 1. chABC cleavage of chondroitin sulfate A to produce the reducing disaccharide (detected by WST-1) and the 4,5-unsaturated disaccharide product (detected by measuring the absorbance at 232 nm). DMMB will bind to large intact proteoglycans.

The most notable of these is that the enzyme is thermally sensitive and almost all of its activity is lost within 3-5 days at 37 °C.⁷ In order to develop better methods of delivery of chABC, given the potential of chABC as a therapeutic intervention, the ability to quantify and accurately assay its activity in model systems is of paramount importance. This is especially important in comparing efficacy of treatments given the broad range in the persistence of enzyme activity seen with commercially purchased chABC batches.⁷

The enzyme commonly used in these studies is chABC from *Proteus vulgaris*. It has broad substrate specificity degrading chondroitin, chondroitin-4-sulfate (also known as chondroitin sulfate A, CS-A), chondroitin-6-sulfate, dermatan sulfate, and hyaluronan by β -elimination of 1,4-hexosaminidic bonds to produce Δ 4,5-unsaturated disaccharides and a reducing end polysaccharide^{8,9} (Figure 1), overall acting as a GAG lyase. Methods previously used for the measurement of chABC activity have been largely based around the use of radioactive substrates,⁹ FACE,^{10,11} HPLC^{7,12} or through indirect detection methods using metachromatic dyes such as Alcian Blue or 1,9-dimethylmethylene blue (DMMB).¹³⁻¹⁶ These techniques have been used for the analysis of CSPGs in urine samples, cartilage culture media, mast cell heparin and chromatographic column sections containing GAGs and proteoglycans.¹⁴ Methodologies based around the use of dyes such as Alcian blue or more recently DMMB have been the most popular assays used for chABC detection, due to their relative speed, ease of use and safety. However despite these advantages, these methods suffer from limitations. The measurements obtained give amounts of total sulfated GAGs present, and therefore are an indirect measurement of chABC activity. These assays also are affected by polyanions, including other sulfated GAGs, DNA and RNA, resulting in positive interference. Furthermore, there are limitations of these assays surrounding their quantitative detection limits and stability of the formed complexes.^{14,15} A further method that has been used for detection of chABC activity involves measuring the formation of the C4-C5 double bond in the product material at 232 nm (Figure 1). Unfortunately, this method also suffers from problems associated with potential interferences at this wavelength and these problems are compounded by the fact that alkenes are relatively weak chromophores.⁹

One method that has received some attention for the detection of biological products¹⁷ is the use of tetrazolium salts that produce highly coloured formazan dyes under reducing conditions. The compound of choice, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), is used in cell metabolism kits (Roche), cell viability assays¹⁸ and for measuring anti-inflammatory responses.^{19,20} To date though, the use of this system in assaying for carbohydrate-processing enzyme activity has only received limited attention.²¹ The use of WST-1 has advantages in that it is a direct measure of enzyme activity due to its ability to form water-soluble complexes that result in a stable complex suitable for accurate spectrophotometric measurements. Spectrophotometric detection is achieved through direct incubation of the WST-1, which in turn reacts with the newly formed reducing disaccharide found only in the product (Figure 1), reducing the WST-1 to WST-1

formazan.²¹ To date however a rigorous investigation into the use of WST-1 for measuring chABC activity has not been conducted. If this method was amenable to measuring chABC activity then a potentially more sensitive and accurate measurement of chABC would be available that could be used to give valuable insight into developing methods for delivery of chABC for treating CNS injury. Here we compare three assays used for chABC activity measurements, demonstrating that WST-1 is a viable, sensitive and reliable alternative to the more classical methods for the measurement of chABC activity in model systems.

Results and Discussion

The three spectrophotometric assays tested included the use of DMMB for the measurement of intact CSPGs present, the evolution of alkenes and in turn the absorbance signal at 232 nm produced from enzymatic activity and finally the novel WST-1 based assay. Initially the assays were compared for their sensitivity to changes in the concentration of chABC against a fixed substrate concentration (chondroitin sulfate A, CS-A, 10 μ M) and time of incubation (2 hours). Treatment of CS-A with a range of chABC concentrations resulted in contrasting results. It was clearly evident that the response for the WST-1 assay was of higher order with regards to linearity and response sensitivity in comparison to both the 232 nm assay and the DMMB assay. This was indicated by the strongest linear correlation coefficient as well as the largest response to increases in enzyme concentration (Figure 2). This linearity is presumably a result of the newly formed hemiacetal from the chABC reaction directly reducing the WST-1 to its coloured formazan (Figure 1). The steeper gradient suggests the range and sensitivity of the WST-1 assay was superior to the other two assays compared in this study.

Figure 2. ChABC standard curves measured with variable enzyme concentration and a constant CS-A concentration of 10 μ M. A) WST-1 assay, B) Absorbance at 232 nm, C) DMMB assay. Results are shown as mean $\pm$ standard deviation of three replicates, significance from background levels determined by ANOVA and Bonferroni-Dunn post-hoc tests (Significance of $P \leq 0.05$).

A more rigorous statistical analysis (Bonferroni-Dunn post-hoc

test ($P \leq 0.05$) was performed to determine at which concentration each assay could detect a significant difference from the background i.e. no enzyme present in the system. Using the WST-1 assay a significant difference could be detected at an enzyme concentration of 12.5 mU/ml, whilst detection using the absorbance at 232 nm assay only was significantly different from background at 50 mU/ml. Surprisingly the DMMB assay showed no significant difference from background levels even at 200 mU/ml, despite an apparent trend in the data (Figure 2).

In an effort to confirm the reliability of the WST-1 assay against a variety of enzyme concentrations, similar experiments were conducted at a range of chABC concentrations over increasing incubation times. The rate of product formation and in turn chABC catalysed CS-A cleavage was linear for concentration ranges between 5 – 50 mU/ml chABC for up to 6 hours of digestion (Figure 3). These results indicate that the assay is not substrate limiting or experiencing any feedback inhibition as a result of product accumulation within the system. Furthermore the direct relationship evident for all concentrations tested indicates, as expected, that the cleavage of CS-A is proportional to the amount of chABC present in the system.

Figure 3. ChABC does not experience substrate inhibition as measured using the WST-1 assay. Varying chABC concentrations against a constant substrate concentration (CS-A, 20 μ M). Results are shown as mean $\pm$ standard deviation of three replicates.

With the lower limits of detection of enzyme activity against a fixed substrate concentration determined, we felt it was important to measure assay sensitivity with samples containing small amounts of substrate (CS-A). We incubated a range of low CS-A concentrations (0.01 – 0.5 μ M) with a fixed concentration of chABC (50 mU/ml). The concentration of enzyme chosen was based on its ability to produce significant digestion after 2 hours of incubation when tested with the WST-1 assay, the absorbance at 232 nm assay and the DMMB assay. Once again the WST-1 based assay provided the best sensitivity (Figure 4A). Using the same statistical analysis as above we found that no values tested in either the DMMB assay or by measuring the absorbance of samples at 232 nm were significantly above baseline levels (Figure 4B,C). The WST-1 assay however showed significance at all concentrations of substrate greater than 0.05 μ M CS-A.

Table 1. Comparison of chABC kinetic parameters. Values are calculated as mean $\pm$ standard deviation from triplicate measurements. Prabhakar *et al.* data is given in Reference 22.

Assay method	K_m (μ M)	k_{cat} (min^{-1})
WST-1 Assay	8.01 ± 0.99	3123 ± 456
232 nm assay	14.85 ± 7.26 $(1.5 \pm 0.1)^{22}$	3848 ± 1339 $(52000 \pm 1300)^{22}$

Now that we had demonstrated the lower detection limits of the enzyme and substrate at fixed substrate and enzyme concentrations respectively, we felt it was important to determine the usefulness of the WST-1 assay for assessing kinetic parameters of the enzyme. This is important from the perspective of using chABC as a therapeutic intervention against CNS injury as differences between enzyme batches and substrate preference can be determined prior to use, as well as enzyme activity can be monitored in time course experiments. Due to its mode of action the WST-1 assay has the advantage that it can be used for the measurement of chABC kinetic parameters. Using this assay the substrate dependence of chABC activity was investigated and found to conform to Michaelis-Menten kinetics (Figure 5). The K_m ($8.01 \pm 0.99 \mu$ M) determined using the WST-1 assay was within reasonable comparison to values determined by the absorbance at 232 nm method and those published within the literature (Table 1).

Figure 4. Comparison of assay substrate detection limits with CS-A. A range of low substrate concentrations (CS-A, 0.01 – 0.5 μ M) were incubated with a fixed chABC concentration (50 mU/ml), with substrate digestion measured by A) WST-1 assay, B) Absorbance at 232 nm assay and C) DMMB assay. Results are shown as mean $\pm$ standard deviation of three replicates. Significance of the results against background levels was determined with ANOVA and Bonferroni-Dunn post-hoc tests (Significance of $P \leq 0.05$.)

However the values for the k_{cat} determined by the WST-1 assay ($3123 \pm 456 \text{ min}^{-1}$) and by the absorbance at 232 nm ($3848 \pm 1339 \text{ min}^{-1}$) method for the chABC sample used in this study was much lower than those published previously using the absorbance signal at 232 nm method (Table 1).²² The differences in k_{cat} could be attributed to the fact that the previous study

assessed a highly purified and freshly prepared chABC sample using the absorbance at 232 nm assay in comparison to the chABC used in this study. A limitation in the DMMB assay is that due to its method for determining activity of chABC it is unsuitable for the determination of kinetic parameters for the enzyme and hence was not compared in these experiments.

Figure 5. Michaelis-Menten kinetic analysis (*inset* Lineweaver-Burk plot) of chABC with CS-A as substrate using the WST-1 based assay.

Although strong evidence of therapeutic benefit has been reported using chABC for treatment of CNS injury, questions remain with regards to the persistence of enzyme activity at 37 °C.^{7,23} The disaccharide trehalose has long been shown to have a positive effect on enzyme stability in both resisting denaturation due to heat treatment as well as during the process of lyophilisation.^{24,25} Despite considerable debate with regards to the mechanism by which trehalose is able to stabilise enzymes,²⁵⁻²⁷ positive effects have been seen with this compound and chABC.²³

Figure 6. Stabilising effects on chABC due to 1 M trehalose. Activity of samples measured by A) WST-1 assay, B) Absorbance measured at 232 nm, C) DMMB assay. Results are shown as mean $\pm$ standard deviation of three replicates. Statistical analysis was performed to test significance of means differing at each time point tested with an ANOVA and Bonferroni-Dunn post-hoc test ($P \leq 0.05$).

Here we tested the ability of 1 M trehalose to stabilise chABC at 37 °C and improve persistence of activity, comparing each of

the assays for the ability to detect any loss in activity over time (Figure 6). It was evident that 1 M trehalose significantly preserved chABC activity at 37 °C, consistent with findings reported in the literature²³ and evident to varying degrees in each of the assays compared (Figure 6). This was also confirmed by SDS-PAGE using decorin as a substrate to directly show the degradation of sulfated GAG side chains from a core protein (Supporting Figures S1 and S2). Once again the best sensitivity was seen with the WST-1 assay which showed significant stabilisation from day 5 onwards when comparing the trehalose stabilised samples to the control chABC at the same time points. The measurement of alkene formation at 232 nm showed a similar result but with significant stabilisation only detected from day 10 onwards. Interestingly, using the DMMB assay resulted in similar trends but no conclusive significant differences were observed between the trehalose stabilised samples and that of the control chABC samples, despite clear evidence from the SDS-PAGE experiments that demonstrate loss of chABC activity in the absence of trehalose (Supporting information S1, S2).

Conclusions

The use of chABC has been demonstrated to be a potentially viable therapeutic option for degrading CSPGs at the site of a CNS injury.^{6,23,28} As a result, useful biochemical assays need to be developed to measure the effectiveness of the enzyme in a variety of conditions. We have demonstrated that the water soluble tetrazolium dye WST-1 is an effective reagent for the detection of reducing end sugars produced by chABC induced enzymatic cleavage of suitable biological substrates. In comparison to other spectrophotometric assays used for the measurement of chABC activity, the WST-1 based assay provides superior results with regards to response and sensitivity at low levels of enzyme and substrate. This assay can also be used to measure the kinetic parameters of chABC as well as the direct thermal stabilisation of chABC in the presence of trehalose. The WST-1 assay may be overall a more general strategy and will find utility in the analyses of chABC activity for industrial and biotechnological purposes.

Experimental

Materials

Chondroitinase ABC (chABC) from *Proteus vulgaris*, chondroitin sulfate A from bovine trachea, trehalose, 1,9 dimethyl methylene blue (DMMB) were used as received from Sigma Aldrich. The tetrazolium salt WST-1 [2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium, monosodium salt] was used as received from Santa Cruz Biotechnology, inc.

WST-1 chABC assay

Assay conditions consisted of 90 μ l substrate at the desired concentration (CS-A prepared in PBS pH 7.4, 0.01 μ M – 270 μ M) with 10 μ l chABC (5 mU/ml – 200 mU/ml) added at $t = 0$. Samples were incubated for 2 hours at 37 °C before being quenched with 100 μ l WST-1 working reagent (1.1 mg/ml WST-1 prepared in 0.1 M NaOH). Samples were then transferred to an incubator at 60 °C for 1 hour to allow for colour development

before measuring the absorbance of the samples in a 96-well plate at 595 nm on an Enspire 2300 multimodal plate reader.

DMMB chABC assay

Assay conditions consisted of 90 µl substrate at desired concentration (CS-A prepared in PBS pH 7.4, 0.01 µM – 270 µM) with 10 µl chABC (5 mU/ml – 200 mU/ml) added at $t = 0$. Samples were incubated for 2 hours at 37 °C before 50 µl of reaction mixture was transferred to wells of a 96-well plate. To each well, 150 µl of DMMB working reagent was added (prepared according to previously described methods¹⁴) and the absorbance at 525 nm was immediately measured within 15 seconds of the addition of working reagent.

Absorbance at 232 nm chABC assay

Assay conditions consisted of 90 µl substrate at desired concentration (CS-A prepared in PBS pH 7.4, 0.01 µM – 270 µM) with 10 µl chABC (5 mU/ml – 200 mU/ml) added at $t = 0$. Samples were incubated for 2 hours at 37 °C before being heat treated at 90 °C for 10 minutes to halt the reaction. Sample absorbance was then measured using a NanoDrop UV-Vis instrument set at 232 nm.

Substrate inhibition experiment

chABC at varying concentrations (10 µl at 5, 10, 25, 50 mU/ml), was combined with 90 µl of 20 µM CS-A at $t = 0$ hours. Samples were incubated at 37 °C for 0.5, 1, 2, 4 or 6 hours before being quenched with WST-1 working reagent and assayed for activity as described above. All assays were performed in triplicate.

Kinetic analysis WST-1

Concentrations of CS-A were prepared in PBS pH 7.4 and varied from 0 – 270 µM with 90 µl of solution per tube. chABC (10 µl, 50 mU/ml) prepared in PBS pH 7.4 was added to each tube in a staggered arrangement to ensure a consistent time of incubation for each sample. Samples were incubated together for 2 hours at 37 °C before being quenched with WST-1 and assayed for activity according to the procedure outlined above. Path length was determined based on manufacturers dimensions of the 96 well-plate (Greiner Bio-One Cell Star), with the WST-1 extinction coefficient calculated from a standard curve (Supporting information S3.)

ChABC versus trehalose treated chABC

ChABC samples of final concentration 200 mU/ml were either prepared in PBS pH 7.4 (chABC control) or in 1 M trehalose solution in PBS pH 7.4, from a chABC stock of 10 U/ml. Samples were heat treated at 37 °C for 0.5, 10, 15 or 20 days and then were immediately frozen (-40 °C). The samples were then thawed together on ice and assayed for activity following the three protocols described above. For analysis of activity against decorin, 10 µl aliquots of the enzyme samples were combined with decorin (10 µl, 800 µg/ml, 8 µg) and incubated together at 37 °C, for 4 hours. After reaction the samples were prepared and separated on a 10% SDS-PAGE gel following standard protocols.

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Notes and references

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- † Electronic Supplementary Information (ESI) available: Activity loss of chABC samples against decorin either with or without trehalose stabilised enzyme assessed by SDS-PAGE and WST-1 standard curve used for the determination of the molar absorptivity of WST-1 at 595 nm. See DOI: 10.1039/b000000x/
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An Improved Assay for the Spectrophotometric Determination of Chondroitinase ABC Activity

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Supporting Information

Figure S1 – SDS-PAGE analysis of degradation products from heat treated chABC alone, with decorin as substrate (i.e. no trehalose added). A) Ladder, B) 0 days, C) 5 days, D) 10 days, E) 15 days, F) 20 days chABC heat treatment at 37 °C, G) decorin with PBS substrate control and H) chABC with PBS control. Dense bands at approximately 55 kDa correlate to the decorin core protein. A diffuse band correlates to the native sulphated glycosaminoglycan side chain decorated core protein illustrated by lane F and G, 20 days of chABC heat treatment and control decorin respectively.

Figure S2 – SDS-PAGE analysis of degradation products from heat treated chABC in the presence of trehalose, with decorin as substrate. A) Ladder, B) 0 days, C) 5 days, D) 10 days, E) 15 days, F) 20 days chABC + trehalose heat treatment at 37 °C, G) chABC with trehalose control and H) decorin with trehalose substrate control. Dense bands at approximately 55 kDa correlate to the decorin core protein. A diffuse band correlates to the native sulphated glycosaminoglycan side chain decorated core protein illustrated by lane H decorin + trehalose control.

Figure S3 – Standard curve used for the calculation of WST-1 molar absorption coefficient.