Anal Bioanal Chem
DOI 10.1007/s00216-015-9222-0

RESEARCH PAPER

"Reagent-free" L-asparaginase activity assay based on CD spectroscopy and conductometry

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Received: 23 August 2015 / Revised: 5 November 2015 / Accepted: 25 November 2015

Abstract A new method to determine the catalytic parameters of L-asparaginase using circular dichroism spectroscopy (CD spectroscopy) has been developed. The assay is based on the difference in CD signal between the substrate (L-asparagine) and the product (L-aspartic acid) of enzymatic reaction. CD spectroscopy, being a direct method, enables continuous measurement, and thus differentiates from multistage and laborious approach based on Nessler's method, and overcomes limitations of conjugated enzymatic reaction methods. In this work, we show robust measurements of L-asparaginase activity in conjugates with PEG–chitosan copolymers, which otherwise would not have been possible. The main limitation associated with the CD method is that the analysis should be performed at substrate saturation conditions (V_{max} regime). For K_M measurement, the conductometry method is suggested, which can serve as a complimentary method to CD spectroscopy. The activity assay based on CD spectroscopy and conductometry was successfully implicated to examine the catalytic parameters of L-asparaginase conjugates with chitosan and its derivatives, and for optimization of the molecular architecture and composition of such conjugates for improving biocatalytic properties of the enzyme in the physiological conditions. The approach developed is potentially applicable to other enzymatic reactions where the spectroscopic properties of substrate and product do not enable direct measurement with absorption or fluorescence spectroscopy. This may include a number of amino acid or glycoside-transforming enzymes.

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Keywords L-Asparaginase · PEG–chitosan · Branched copolymers · Catalytic activity · Conductometry · CD spectroscopy

Introduction

Bacterial L-asparaginases are used in amino acid depletion therapy of acute lymphoblastic leukemia in children [1–4]. Additionally, there are new medical applications being studied, including Hodgkin's disease, melanosarcoma, and multiple myeloma [5, 6]. However, the use of L-asparaginases in oncotherapy is limited by serious side effects, mostly related to the bacterial enzyme's immunogenicity. Another problem limiting the efficiency of the treatment is the fast enzyme biodegradation in blood [4, 5]. The same applies to many other therapeutic enzymes and biotherapeutics, limiting their efficiency and patient compliance. Thus, better understanding of structure–function relationship in such systems, as well as new approaches for their targeted manipulation, is of high interest from both fundamental and practical standpoints [7–10].

With this in mind, we have synthesized the range of PEG–chitosan copolymers of different compositions with the purpose of studying their conjugates with L-asparaginase. Physicochemical and, accordingly, biological properties of chitosan-conjugated enzyme can be varied within a broad range depending on the degree of polymerization, polyelectrolyte charge density, and the presence of substituents (including PEG molecules) in the polymer chain of chitosan, allowing the regulation of catalytic properties of the enzyme [11, 12]. Moreover, it should be expected that the use of branched polymers would result in lower immunogenicity of enzyme preparations, which might eliminate some restrictions on therapeutic application of bacterial preparations.

Published online: 16 December 2015

However, the lack of an appropriate activity assay for L-asparaginase has almost barred such studies. Up to now, the main activity assay for L-asparaginase is based on Nessler's method [13, 14] which suffers from several limitations. First, it is multistage and the calibration experiments are required for each set of measurements; thus, it is laborious and does not work well in screening mode. Second, and this is even more important, Nessler's reagent is not selective enough for the ammonium ion and a number of compounds, such as guanidines, primary and secondary alcohols, amines, aldehydes, etc., interfere with the reaction [14]. The conjugated enzymatic reaction assay recently described elsewhere [15] has limited applicability for colloidal or micro-heterogeneous systems (such as, for example, reverse micelles), as well as for complex multi-component systems, containing polyelectrolytes, affecting the assay.

In the present study, circular dichroism spectroscopy (CD spectroscopy) is developed as a direct method to determine catalytic activity of L-asparaginase. L-Amino acids are known to be optically active compounds, and the difference in molar ellipticity between the substrate (L-asparagine) and the product (L-aspartic acid), as measured by CD spectroscopy, enabled reliable a V_{max} assay for native L-asparaginase, as well as for its conjugates with PEG–chitosan.

As a complimentary method to determine the catalytic parameters of L-asparaginase, the conductometry is suggested in this study. Conductivity increases in the reaction system due to the release of ammonium and aspartate ions upon L-asparagine hydrolysis catalyzed by L-asparaginase. However, conductivity is much less robust compared to CD spectroscopy and strongly depends on many factors, such as buffer composition and ionic strength, thus requiring thorough calibrations and controls. Although this method is not as rapid as CD spectroscopy, it allows for measurement in heterogeneous and non-transparent systems. Conductometry, similar to CD spectroscopy, is a "reagent-less" method which enables continuous measurements.

Therefore, this study was focused on (i) development and validation of the two complimentary methods to determine the catalytic parameters of L-asparaginase preparations (by comparing with Nessler's method) and (ii) application of the two complimentary methods in development of L-asparaginase conjugates with charged amino-polysaccharides (which is not possible with Nessler's method). We believe the enzyme activity assay based on CD spectroscopy is potentially applicable to many other amino acid or glycoside-transforming enzymes, including that in complex mixtures and conjugates.

Materials and methods

Materials

The preparation of recombinant L-asparaginase from *Erwinia carotovora* (EwA) was obtained by the method described previously [16]. Chitosan (5 and 15 kDa) were from Sigma (USA) and Bioprogress (Russia). Monomethoxypolyethyleneglycol-*N*-hydroxysuccinimidyl-succinate (mPEG-suc-NHS, 5 kDa) was kindly provided by N.S. Melik-Nubarov (Chemical Faculty, M.V. Lomonosov MSU). Other compounds such as DMSO and components of buffer solutions were from Sigma.

Synthesis of PEG–chitosan copolymers and their conjugates with L-asparaginase

PEG–chitosan copolymer was synthesized using mPEG-suc-NHS as activated PEG derivative [17, 18]. The conjugates of L-asparaginase with PEG–chitosan were synthesized by modification of amino groups of L-asparaginase via reductive amination [17, 18]. The purity of the preparation was controlled by HPLC gel filtration in a Knauer chromatography system (Germany) on BioFox 17 SEC in a 15 cm×1 cm^2 column. The eluent was 15 mM PBS, pH 7.5, 150 mM NaCl; the elution rate was 0.5 ml/min, 25 °C.

L-Asparagine and L-aspartic acid CD spectra

The asparagine and aspartic acid CD spectra were recorded with a Jasco J815 circular dichroism spectrometer. The measurements were made in the wavelength range 195–220 nm at 37 °C in a 1-mm pathlength quartz cuvette. The spectra were obtained by fivefold scanning with 1-nm steps. L-Asparagine and L-aspartic acid were dissolved in 15 mM PBS buffer, pH 7.5; the amino acid concentration was 3–20 mM.

Determination of L-asparaginase catalytic activity by CD spectroscopy

The rate of asparagine hydrolysis was measured with a Jasco J-815 circular dichroism (CD) spectrometer (Japan) with a temperature-controlled cell. In a typical experiment, EwA solution in 15 mM phosphate-buffered saline was added to the L-asparagine solution in the same buffer so that the resulting mixture would contain 0.002–0.015 mg/ml of the enzyme and 20 mM L-asparagine. The reaction was carried out at 37 °C in a 1-mm pathlength quartz cuvette. The time-dependent ellipticity was recorded at 210 nm.

Determination of L-asparaginase catalytic activity by conductometry

The rate of asparagine hydrolysis was measured with a Mettler Toledo T-70 automatic titrator equipped with a conductometry sensor InLab© 741 (Switzerland). In a typical experiment, an aliquot of EwA in 15 mM PBS buffer was added to the L-asparagine solution in the same buffer. The L-asparagine concentration was 0.1–2 mM. The reaction was carried out at

37 °C. To determine the enzymatic reaction rate values, the calibration dependence of conductivity on the system composition corresponding to substrate conversion degrees from 0.5 to 10 % was used.

Results and discussions

Determination of L-asparaginase catalytic activity by CD spectroscopy In the present study, the CD spectroscopy method is suggested to determine catalytic activity of recombinant L-asparaginase from *E. carotovora* (EwA) and its modified forms. Both L-asparagine and L-aspartic acid are optically active in the far UV region (195 to 215 nm) due to the presence of carboxylic and amide groups. However, the molar ellipticity of the product is much higher than that of the substrate (Fig. 1a). The wavelength of 210 nm was chosen to ensure robust measurements at optimal signal–noise ratio. Linear calibration dependences of the CD signal (ellipticity θ) at 210 nm were obtained for a broad range of L-asparagine and L-aspartic acid concentrations (Fig. 1b). These calibration curves were used further in this study to analyze the enzymatic activity of L-asparaginase.

To determine the substrate saturation conditions (for V_{max} measurement), the initial reaction rate dependence on the L-asparagine concentration was obtained (Fig. 2). The reaction rate rises sharply with an increase in substrate concentration up to 5 mM followed by an inflection point and then by a classical plateau: at an L-asparagine concentration of 15–20 mM and higher, substrate saturation is observed (Fig. 2). The concentration of 20 mM was used in further experiments for determination of V_{max} parameter for L-asparaginase preparation. For this substrate concentration, the dependence of the catalytic activity is found to be proportional to enzyme content in the wide range of enzyme concentration from 0.001 to 0.015 mg/ml (Fig. 3a), indicating validity of the activity assay.

L-Asparaginase specific activity determined from this dependence is 490 (±30) IU/mg, which is in a good agreement with the value obtained by Nessler's method 470±30 IE/mg. The correlation coefficient between the two methods was found to be 0.94, as determined from the correlation dependence at different enzyme concentrations (Fig. 3b).

It was demonstrated here that CD is an express and robust method allowing the determination of the V_{max} parameter in the range of the measurement error of 5–10 % depending on the substrate concentration. The CD method appears less sensitive compared to Nessler's method (0.1–1 IU/ml versus 0.02–0.1 IU/ml) [14]. However, unlike Nessler's method, CD spectrometry is applicable for high-throughput activity measurements, which facilitates the whole range of new applications. One may conclude that CD is most appropriate for express measurements of V_{max}. When reaction rate measurements at L-asparagine concentrations lower 1 mM are necessary (for example in the case of K_M determination), the complimentary method is needed, since the sensitivity of the CD method will limit such measurements. Therefore, we have assessed the potential of conductometry-based measurements for more detailed enzymatic kinetics studies.

Determination of L-asparaginase catalytic activity by conductometry The method is based on conductivity increase due to the release of ammonium and aspartate ions upon asparagine hydrolysis catalyzed by L-asparaginase. We have found that in the case of conductometry the optimal experimental conditions for L-asparaginase activity measurement are the substrate concentration interval from 0.1 to 2 mM, allowing the determination of both V_{max} and K_M parameters (since K_M is 0.1–0.2 mM). The V_{max} of 430 (±20) IU/mg and K_M of 0.08 mM for EwA are in a good agreement with those obtained with Nessler's method (V_{max} of 470±30 IU/mg and K_M of 0.12 mM) [7] and with the V_{max} value obtained by CD measurements (V_{max} of 490±30 IU/mg).

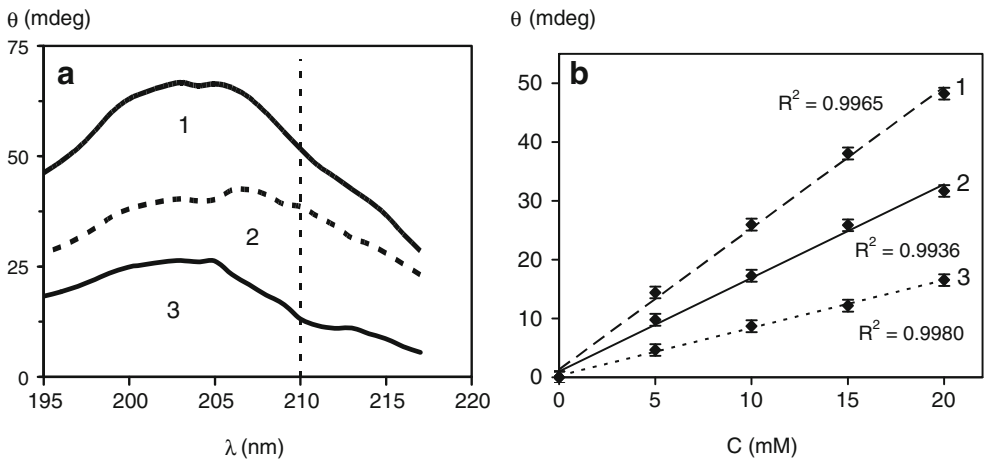

Fig. 1 **a** CD spectra of L-aspartic acid (*1*) and L-asparagine (*3*) and the difference spectrum (*2*); L-aspartic acid and L-asparagine concentration was 20 mM. **b** The calibration dependences of ellipticity (θ at 210 nm) on L-aspartic acid concentration (*1*) and L-asparagine (*3*) and their difference (*2*). Difference molar absorption coefficient is 1.6 mdeg/mM. Experimental conditions: 15 mM potassium–phosphate buffer pH 7.5, 37 °C

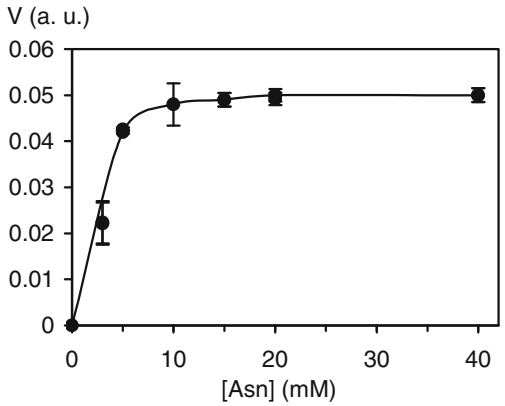

Fig. 2 Dependence of the enzymatic reaction rate on L-asparagine concentration in 15 mM PBS, pH 7.5, 37 °C; enzyme concentration was 10 μg/ml

It is worth mentioning that at substrate concentrations of 5 mM and higher, conductometry measurements are barely possible due to unfavorable signal-to-noise ratio. So, this is truly a complimentary method to CD. The applicability of both CD and conductometry to complex multi-component systems, containing polymers or substances with hydroxylic and amino groups, was studied using EwA activity in conjugates with PEG–chitosan copolymers. Measurements with Nessler's method are compromised in such systems [19], thus complicating the development of new formulations and/or the prolonged action form of the enzyme.

Application of CD and conductometry assays for development and optimization of the catalytic properties of L-asparaginase conjugates with chitosan and PEG–chitosan copolymers

To design new L-asparaginase preparations with improved features, the conjugates of the enzyme with branch copolymers based on chitosan and PEG designated as ChitoPEGylation have been suggested in our earlier reports [18]. An important advantage of the ChitoPEGylation approach is the polyelectrolyte nature of the copolymers, which determines multipoint electrostatic interaction with the protein surface, thereby stabilizing the enzyme conformation, and protecting it from aggregation through electrostatic repulsion of charged particles. Besides, interaction with PEG–chitosan creates a specific microenvironment near the active site of the enzyme; the latter allows regulating catalytic properties of the enzyme, such as shifting pH optima [20].

Here we have applied the newly developed activity assays to obtain the EwA–PEG–chitosan conjugate with optimal biocatalytic properties. For this purpose, the influence of chitosan molecular mass as well as composition of the PEG–chitosan copolymer (the PEGylation degree of chitosan) on the catalytic activity of the enzyme has been investigated.

The catalytic parameters of EwA conjugates with chitosan and PEG–chitosan in comparison with native enzyme measured by CD spectroscopy are summarized in Table 1. The specific activity of EwA conjugates with chitosan depends significantly on both chitosan MW and on conjugate composition (Table 1). So, L-asparaginase conjugate with 5 kDa chitosan shows the highest specific activity which is ~6 times higher as compared to native enzyme, while for EwA–chitosan conjugate with chitosan of 15 kDa, the activity is ~3 times higher compared to native EwA. Further increase in chitosan MW decreased significantly the conjugate activity: for EwA–glycol–chitosan (72 kDa) and for EwA–chitosan (160 kDa), the activity is ~2 times and ~10 times lower correspondingly compared to native EwA.

Variation of chitosan PEGylation degree also has pronounced effect on the enzyme catalytic activity. A number of conjugates of EwA with PEG–chitosan with different PEG content were synthesized based on chitosan with MM of 15 kDa. For this chitosan sample, the PEG–

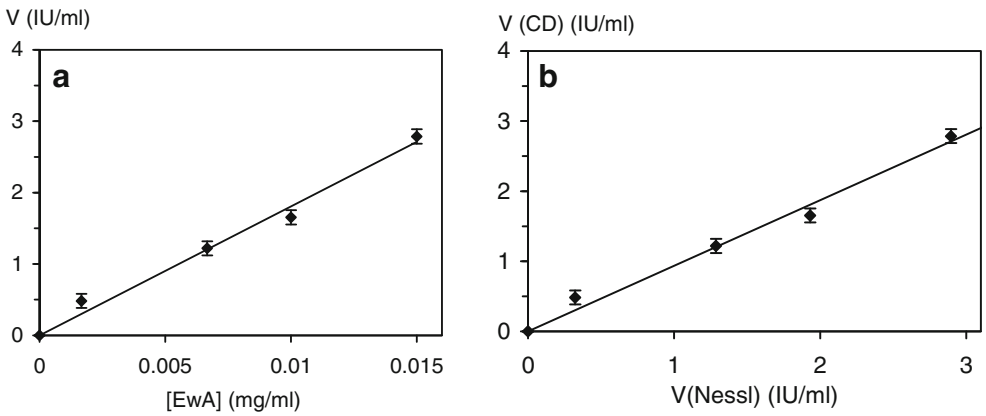

Fig. 3 **a** Dependence of L-asparagine hydrolysis reaction rate on the enzyme concentration at saturating L-asparagine concentration (20 mM). **b** Correlation dependence of asparagine hydrolysis rates determined by CD spectroscopy (V_{CD}) and by Nessler's method (V_{Nessl}) obtained by varying the enzyme concentration

Table 1 Specific activity of EwA and EwA–PEG–chitosan conjugates depending on chitosan MW and PEG to chitosan ratio

Conjugate	Conjugate composition	Specific activity, IU/mg
EwA	–	490 (±20)
EwA–chitosan	EwA–chitosan (5 kDa)	2940 (±20)
	EwA–chitosan (15 kDa)	1284 (±17)
	EwA–glycol–chitosan (72 kDa)	260 (±20)
	EwA–chitosan (160 kDa)	46 (±7)
EwA–PEG–chitosan	EwA–PEG–chitosan (15 kDa), *n*(PEG)/*n*(chit)=2	1668 (±20)
	EwA–PEG–chitosan (15 kDa), *n*(PEG)/*n*(chit)=8	1644 (±17)
	EwA–PEG–chitosan (15 kDa), *n*(PEG)/*n*(chit)=14	1856 (±15)
	EwA–PEG–chitosan (15 kDa), *n*(PEG)/*n*(chit)=20	1950 (±17)
	EwA–PEG–chitosan (15 kDa), *n*(PEG)/*n*(chit)=40	163 (±20)

Experimental conditions: 15 mM PBS buffer, pH 7.5, 37 °C

n(PEG)/n(chit) number of PEG chains per chitosan molecule

chitosan copolymers with wide range of PEGylation degree can be obtained: the PEGylation degree in the PEG–chitosan copolymer was varied in the wide range from 0 to 40 PEG chains per chitosan molecule. Interestingly, the increase in chitosan PEGylation degree (up to *n*(PEG)/*n*-(chit) ratio of 20) results in the increase in the enzyme catalytic activity. The significant activation effect is observed already at *n*(PEG)/*n*(chit) ratio of 2 compared to conjugate with non-PEGylated chitosan (Fig. 4). Only at very high PEGylation degree (at *n*(PEG)/*n*(chit) ratio of 40, which means that more 50 % of amino groups are PEGylated) is the activity decreased. Most probably, at such PEGylation degree, the substrate access to the active site of the enzyme is limited due to steric factor. So, the activity optimum is observed at *n*(PEG)/*n*(chit) ratio of 15–20 where catalytic activity of conjugates increased by the factor of 3–4 in comparison with native enzyme

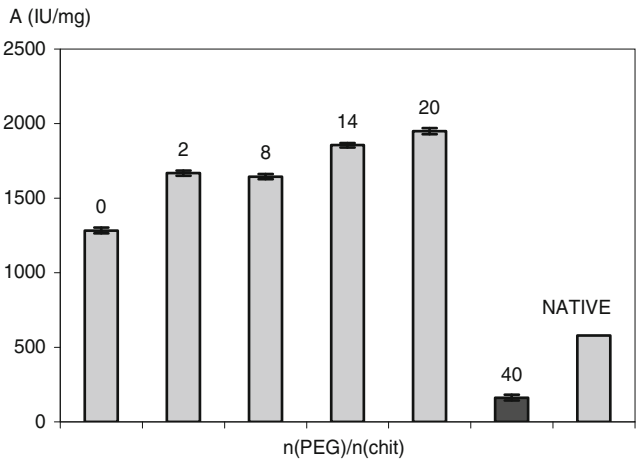

Fig. 4 Dependence of specific activity of EwA conjugates with PEG–chitosan on the chitosan PEGylation degree as compared to native enzyme. 15 mM PBS buffer, pH 7.5, 37 °C. L-Asparagine concentration was 20 mM; enzyme concentration was 10 μg/ml

(Table 1). This activation effect can be explained by the increase in the efficiency of the interaction of the polymeric chain with protein globule with increasing in chitosan PEGylation degree. This can be due to increase in the copolymer chain mobility and copolymer branching degree providing more tight contact of copolymer with the enzyme surface. So, it is known that PEGylation of chitosan leads to higher mobility of polymeric chains, which is manifested through the decrease in the solution viscosity upon chitosan modification with PEG. On the other hand, the activation effect observed for the EwA–PEG–chitosan conjugates is related to the shift of pH optimum towards physiological pH from pH 8.8 (for native EwA) to pH 6.8–7.8 observed for EwA–PEG–chitosan and EwA–chitosan conjugates [17]. The magnitude of the pH optimum shift depends on the number of unmodified amino groups in chitosan polycationic chains which decreases with increase in chitosan PEGylation degree. So, at *n*(PEG)/*n*(chit) ratio of 14–20, the optimal conjugate structure and composition are achieved in terms of polymeric chain mobility, copolymer branching degree, and pH optimum shift. Thus, the optimization of conjugate molecular architecture and composition significantly improved the catalytic activity of the EwA conjugate as compared to the native enzyme under physiological conditions and this approach can be applied for regulation of the enzyme catalytic properties.

Up to now the activation effect was discussed in terms of increase in V_{max} for EwA–PEG–chitosan conjugates. However, formation of the conjugates with branched copolymers (especially at PEG–chitosan with long polymer chains or with high chitosan PEGylation degree) may lead to reduced substrate access to the active site of the enzyme. In this case, the control of the Michaelis constants (K_M) is necessary. These measurements were carried out with conductometry method developed above for native EwA.

Table 2 Kinetic parameters of EwA–PEG–chitosan conjugate in comparison with native EwA determined by conductometry, CD spectroscopy, and Nessler's method

Conjugate	EwA			EwA–PEG–chit (15 kDa)		
Method	A, IU/mg	K_M, mM	A/K_M	A, IU/mg	K_M, mM	A/K_M
Conductometry	430 (±25)	0.08 (±0.01)	5370 (±740)	1800 (±25)	0.12 (±0.01)	15,000 (±1267)
CD	490 (±20)	-	-	1856 (±20)	-	-
Nessler's method	470 (±20)	0.12 (±0.01)	3900 (±470)	-	-	-

- means that the parameter cannot be determined by this method. Experimental conditions as in Table 1

Measurements of Michaelis constants (K_M) and the maximal reaction rate (V_{max}) of EwA conjugates with conductometry

To examine the influence of conjugate formation with chitosan and PEG–chitosan on both the Michaelis constants (K_M) and the V_{max} of EwA, the activity assay based on the conductometry method developed above was applied. The specific activities of the enzyme EwA–PEG–chitosan conjugate (with chitosan of 15 kDa and n(PEG)/n(chit)=14) was 1800 IU/mg, which is in a good agreement with the results obtained by CD spectroscopy method (Table 2).

The specific activity of EwA–chitosan conjugate at n(PEG)/n(chit)=14 was about four times higher compared to the native enzyme (Table 2), while the K_M value for EwA–PEG–chitosan (0.12±0.05 mM) was not much different from those obtained for native EwA (0.08± 0.05 mM). This finding is a promising starting point to apply ChitoPEGylation approach for development of the dosage forms of L-asparaginase with prolonged action. Interestingly, using the conductometry method, it was demonstrated that the K_M value for EwA conjugates with chitosan of high MW (up to 160 kDa) also remained unchanged compared to native EwA, but it shows the significant decrease in the V_{max} value (down to 61 IU/mg), as it was also observed with using CD spectroscopy method for EwA conjugates with polymers based on chitosan of high MW (Table 1). This indicates that the decrease in the enzyme activity for EwA conjugates with high MW is caused rather by the influence of long-chain polycation on the protein conformation then by steric hindrance for the substrate diffusion to the enzyme active center. Thus, optimal catalytic efficiency is observed for EwA conjugates based on chitosan with MW 15 kDa and less.

Thus, application of the activity assay developed based on combination of CD spectroscopy and conductivity allowed finding the optimal structure and composition of L-asparaginase conjugates with chitosan and its copolymer with PEG. We found that branched copolymers of charged polysaccharides with PEG, being attached to enzyme (for example EwA–chit (5 kDa) and EwA–PEG–chit (15 kDa and n(PEG)/n(chit)=14)), improve the L-asparaginase catalytic activity at physiological conditions with a factor of 6 and 3 correspondingly (Tables 1 and 2) in comparison with native enzyme. The above effects if translated in clinic could increase significantly the therapeutic efficiency of L-asparaginase, which means lower dosages and higher tolerability for patients.

It should be mentioned that measurements taken with optical techniques such as CD spectroscopy are very fast and enable continuous measurements. CD spectroscopy due to the possibility of following the reaction in the substrate saturating conditions allows accurately determining the V_{max} parameter. This is crucial when developing, e.g., a new dosage form of the enzyme with improved therapeutic properties. However, when the detailed investigation of catalytic parameters of L-asparaginase preparations is needed, the conductometry method is useful, which, although being more laborious and time-consuming compared to CD spectroscopy, enables more detailed characterization, including both V_{max} and K_m values, and so it can be used as a truly complimentary method to CD.

Conclusions

The combination of CD spectroscopy and conductometry methods provides novel opportunities for high-throughput assay of catalytic properties of L-asparaginase preparations not only with native enzyme but also with its modified forms including conjugates with polymers containing hydroxylic and amino groups, such as chitosan and PEG–chitosan copolymers. This would not have been possible using Nessler's method. This holds promise for the development of prolonged action dosage forms for this therapeutic enzyme. The methods described here could be potentially used for a good number of the enzymes (including medically relevant) where the measurements with existing methods are not always possible, unreliable, and extremely labor-consuming.

Acknowledgments This project was supported by RFBR grants 14-04-00325A, and using equipment purchased in the frames of development programs of Lomonosov MSU 2011–2015.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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