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Quantitative determination of chitinolytic activity of lysozyme using half-deacetylated chitosan as a substrate

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ABSTRACT

In this paper, we report a novel quantitative assay for chitinolytic activity of lysozyme. Our assay is based on measuring the reducing sugar ends (RSE) released from the water-soluble macromolecular substrate, half-deacetylated chitosan. RSE reacts with 3-methyl-2-benzothiazolinonehydrazone to produce the chromogenic product that has absorption maxima at 615 and 655 nm. We found that measurement of low levels of RSE at 655 nm yields higher precision and better linearity than that at 615 nm. The optimal procedures are: the pre-warmed half-deacetylated chitosan and lysozyme were mixed to make the final solution containing 4 mg mL^{-1} half-deacetylated chitosan at pH 4.0 in sodium acetate buffer, and incubated at 40 °C for 1 h within steady state time course. The linear range of lysozyme activity was within 94–1006 nM min^{-1}. We demonstrated that our method is simple, rapid, inexpensive and accurate, which other low-sensitive methods cannot be achieved. This method can also be used to quantitatively determine the activity and kinetic parameters of chitinase and chitosanase.

1. Introduction

Lysozyme (EC 3.2.1.17) is a ubiquitous enzyme occurring in a wide range of biological fluids and tissues within animal and plant kingdoms, such as human serum, urine, tears, seminal fluid, and milk. Lysozyme has been studied extensively as a model protein for structural, physicochemical, crystallographic, enzymatic, immunological, and evolutionary research. Lysozyme uses polysaccharides, which are the main components of bacterial cell wall, as natural substrates. It prefers to hydrolyze substrates with alternating units of N-acetylglucosamine and N-acetylmuramic acid (Chipman, Grisaro, & Sharon, 1967), but it can also hydrolyze the similar glycan backbone, such as chitin, partially deacetylated chitosan (Amano & Ito, 1978; Kristiansen, Vårum, & Grasdalen, 1998; Nordtveit, Vårum, & Smidsrod, 1996; Vårum, Holme, Izume, Stokke, & Smidsrød, 1996), which are polymers of glucosamine and N-acetylglucosamine. Lysozyme has antifungal, antiviral, antitumor and immune modulatory activities (Lee-huang et al., 1999; Samaranayake, Samaranayake, Pow, Beena, & Yeung, 2001; Sava, 1996), which makes lysozyme a safe food preservative as well as an active substance included in pharmaceutical preparations.

Most of the currently used methods for analysis of lysozyme activities are based on the lysis of *Micrococcus lysodeikticus* cells, with their peptidoglycan as a substrate. Detection of bacterial lysis can be achieved by turbidimetric method (Gorin, Wang, & Papapavlou, 1971; Lee & Yang, 2002; Levashov, Sedov, Shipovskov, Belogurova, & Levashov, 2010; Shuga, 1952; Zhao, Zhang, & Yang, 2002), agar diffusion method (Modeer & Soder, 1971; Qin & Peng, 1998), fluorometric method (Hannig, Spitzmüller, & Hannig, 2009; Helal & Melzig, 2008), Resonance scattering spectra method (Jiang & Huang, 2007), and agarose rocket electrophoresis method (Chen, Wang, Zhou, Zhong, & Liu, 1988). However, it should be noted that bacterial lysis is a complex reaction that is mediated by both bacterial enzymes and lysozyme. Assays based on bacteria must be conducted under stringent conditions with respect to concentration of bacterial suspension and the range of absorbance decrease, lytic temperature and the duration of the measurement. These restrictions pose serious problems when kinetic parameters, accuracy, high precision, and reproducibility are required. ELISA methods are also reported (Kerkaert, Mestdagh, & Meulenaer, 2010; Vidal, Gautron, & Nys, 2005) but they are used to detect lysozyme protein, but not lysozyme activity.

A method for the determination of lysozyme activity, based on a single chemical reaction and a pure substrate, has obvious advantages. Isolation of peptidoglycan in a native form is quite difficult because the organisms that are most susceptible to lysozyme are highly resistant to mechanical disintegration. Iso-

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lation and purification of peptidoglycan in their high polymer form had very little success. Therefore, some other compounds such as chitin and its soluble chitin derivatives were investigated as artificial lysozyme substrates: O-hydroxyethylated chitin (glycol-chitin) (Imoto & Yagishita, 1971), carboxymethyl-chitin, dihydroxypropyl-chitin (Maeda, Matsumoto, & Kondo, 1997), chitosan and dye-labled chitosan (Vårum, Myhr, Hjerde, & Smidsrød, 1997; Wirtha & Wolf, 1990). All have the potential to be substrates for lysozyme.

The substrate degradation rate is generally measured by changing in three properties: decrease of viscosity (Nordtveit et al., 1996; Yamaoka, Ishii, Natsume, & Moriyama, 1989), labeled substrate consumption (Xie, 2003), and release of reducing ends (Imoto & Yagishita, 1971). Because viscometric method is relatively tedious and slow and partially hydrolyzed labeled substrate could hardly stay in acid solution, many researchers concentrated on reducing ends determination. The commonly used methods to determine reducing ends are DNS method (Miller, 1959), Somogyi–Nelson's method (Nelson, 1944; Somogyi, 1945) and potassium ferricyanide method (Imoto & Yagishita, 1971; Kurita, Kaji, Mori, & Nishiyama, 2002; Xie, 2003; Zhou, Huang, Tan, Liang, & Zhou, 2002). Potassium ferricyanide method is the most sensitive method of the three, which is based on the disappearance of potassium ferricyanide due to the released reducing groups by lysozyme, and increasing concentrations of reducing ends lead to a decrease in color. But this method has relatively low sensitivity that makes impossible to detect the initial velocity of lysozyme during the enzymatic degradation of substrates because of the relatively high K_m value of lysozyme (we will discuss in this paper), to say nothing of DNS method and Somogyi–Nelson's method. Anthon and Barrett (2002) introduced a more sensitive method, MBTH (3-methyl-2-benzothiazolinonehydrazone) method, for the measurement of reducing sugars. Horn and Eijsink (2004) reported that the standard curves for the chito-oligosaccharides measured by the MBTH method were independent of the length of the chito-oligosaccharide. However, the substrates (N-acetylglucosamine$_4$ and N-acetylglucosamine$_6$) they used have relatively low molecular weight and the substrate itself comprise a high proportion of reducing ends. As a result, the color of the blank solution is too dark for the spectrophotometer to be adjusted to zero in the activity measurement. On the other hand, these substrates are expensive and thus can be hardly utilized for the commercial uses.

In this article, we describe a novel spectrophotometric method to quantitatively detect chitinolytic activity of lysozyme. We use easy-to-prepare and low-cost half-deacetylated chitosan with high molecular weight (>1 million Dalton) (Zhang, Xue, Xue, Li, & Fu, 2006) as a substrate and use MBTH to detect the reducing sugar ends released by lysozyme. The method was validated in terms of linearity, quantitation limit, detection limit, precision, and accuracy. Our method is simple, low-cost, and highly accurate.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white in the form of lysozyme chloride (15,300 U/mg, measured with turbidimetric method, protein 90%) was purchased from Sigma–Aldrich (L2879). One unit (1 U) of lysozyme activity was defined by the manufacturer as decreasing a 0.001 absorption value at 450 nm per minute at pH 6.24, 25 °C, and using suspension of *M. lysodeikticus* as a substrate, in a 2.6 mL reaction mixture (1 cm light path). Lyophilized cells of *M. lysodeikticus* and lysozyme standard were from Nanjing Jiancheng Bioengineering Institute. Half-deacetylated chitosan was derived from shrimp α-chitin by strong alkaline deacetylation (Zhang, Xue, Xue, Gao, & Zhang, 2005; Zhang et al., 2006), and its degree of deacetylation determined by ^{1}H NMR (Lavertu et al., 2003) was 52.4%. 3-Methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH), 1,4-dithiothreitol and N-acetylglucosamine were from Sigma–Aldrich. All other chemicals were analytical grade, and distilled water was used.

2.2. General assay for the reducing sugar (MBTH method)

The procedure (Anthon & Barrett, 2002) was appropriately modified according to the properties of the half-deacetylated chitosan and its enzymatic degradation. 0.5 mL sample containing N-acetylglucosamine or substrate hydrolytes was mixed with 0.5 mL 0.5 M NaOH in a test tube (15× 100 mm). Equal volumes of 3 mg mL^{-1} MBTH and 1 mg mL^{-1} 1,4-dithiothreitol were mixed and 0.5 mL of this fresh mixture were added to the test tube, and heated for 15 min at 80 °C in a water bath with thermostat. Immediately after removal from the water bath, 1 mL of a solution containing 0.5% ($FeNH_4(SO_4)_2$)·$12H_2O$, 0.5% sulfamic acid and 0.5 N HCl was added to the samples. The tube was sealed with a membrane, inverted and mixed thoroughly with vortex, and allowed to cool to room temperature. The absorbance was measured at 655 nm by a UV-VIS spectrometer UV2550 (SHIMAZU), with a 0 μg mL^{-1} reducing sugar sample as a blank solution. All data from the reducing sugar assay are expressed in terms of reducing sugar end equivalents generated relative to N-acetylglucosamine as a standard. All assays were performed in triplicates.

2.3. Spectra scanning and calculation for calibration line and precision on each wavelength

Three repetitions of ten different concentrations (0–20 μg mL^{-1}, prepared from its stock 10 mg mL^{-1} solution) of N-acetylglucosamine were determined according to the MBTH method described above. In order to confirm the optimal wavelength for the measurement, the spectra were taken with a slit width of 2 nm, from 500 to 750 nm at 1 nm increments, using 10 mm path length cuvettes. The correlation coefficients (R^2) and slopes of the regression lines, and the relative standard deviation (RSD) of each concentration level (n = 3) in every 1 nm were calculated using Microsoft Excel (Microsoft Corporation).

2.4. General assay for chitinolytic activity of lysozyme

Half-deacetylated chitosan was dissolved in 50 mM sodium acetate buffer to make the final solution at pH 4.0, using pH meter equipped with LE 420 electrode (FiveEasy^{TM}, METTLER TOLEDO).

In order to optimize the method, multiple points of sampling method were used. A given volume (e.g. 5 mL) of chitosan solution in a conical flask was incubated in a shaking water bath (±0.1 °C, 100 rpm) to a given temperature, added with equal volume of lysozyme solution which was prewarmed to the same temperature, and then mixed thoroughly to start the reaction (t = 0 min). 2 mL aliquots of the incubated reaction mixture were periodically taken and immediately stopped by adding to the same volume of 0.5 M NaOH and shaking vigorously. Three replicates were performed and their reducing sugar contents were analyzed according to the previous section. Initial reaction velocities were obtained from the slope of the best fit to the initial linear portion of the product progress curve.

A pH-dependent assay was performed at 40 °C with buffers in the range of pH 3–7. Half-deacetylated chitosan was dissolved in 50 mM sodium acetate buffer to make (if necessary, small volumes of concentrated HCl or NaOH were used) the final solution at desired pH, using the lysozyme assay described above.

In the experiments where temperatures varies, 0.1 mL lysozyme solution was mixed with 9.9 mL half-deacetylated chitosan solution (4 mg mL^{-1}, pH 4.0) which was pre-warmed to the desired temperature (30–90 °C), using the lysozyme assay described above.

The apparent K_m value of lysozyme was determined according to the method in the previous section with varying the final concentrations of half-deacetylated chitosan from 0.625 to 6 mg mL^{-1} and a fixed amount of lysozyme. The kinetic constant was obtained by fitting the experimental data to the appropriate rate equations using linear regression according to Lineweaver–Burk plot.

In order to validate the method, an end point of sampling method was used. 0.25 mL of chitosan solution was mixed with equal volume of lysozyme solution to start the reaction for 1 h, and stopped with 0.5 mL 0.5 M NaOH solution. Substrate blank was used in order to offset the interference of the non-enzymatic hydrolysis of the substrate during the incubation time with the mesurement. Triplicates were performed and analyzed as described above.

2.5. Method validation

The analytical characteristics of the method were validated to ensure the suitability of the analytical requirements and the reliability of the results. The relationship between the concentration of lysozyme and absorbance at 655 nm was determined to be linear. The correlation coefficient (R^2) was calculated by means of least-squares analysis, using three repetitions of eight different final concentrations (0, 1, 2, 3, 4, 5, 6, 8 μg mL^{-1}, expressed as turbidimetric method: 15.3 U/μg). The detection limit (DL) and quantification limit (QL) were calculated from the calibration lines that define its linearity (Miller, 1991). They were calculated according to DL = $3 \times S_{b1}/b$, and QL = $10 \times S_{b1}/b$, Where S_{b1} is the standard error of the absorbance of the blank solution (ten replicates), and b is the slope of the calibration lines. The precision of the method indicates the degree of dispersion obtained with a series of determinations on the same sample. Six measurements were performed for samples containing 76.5 U mL^{-1} (5 μg mL^{-1}) of the enzyme. The relative standard deviation (RSD) for the Horwitz criterion (CIPAC, 1999) was evaluated. The acceptability of the results is based on the modified Horwitz equation: RSDr < $2^{(1-0.5\log C)} \times 0.67$, where C is the average concentration of the analyte ($\times 10^{-6}$ μg mL^{-1}) in the sample as a decimal fraction.

Accuracy shows the proximity between the experimental and theoretical values. The determination of this parameter was performed by studying the recovery after standard addition procedure using two addition levels (46 and 92 U lysozyme mL^{-1}) based on the lysozyme sample containing 30.6 U mL^{-1}. Six determinations were carried out for each addition level and the % of recovery was calculated. The homogeneity of variances of the measurements for each assayed level was verified by a Cochran test (Bao, Bao, & Niu, 2006). The mean recoveries of each level were compared using a Student's t-test by comparing the experimental t-value (t_{exp}) to the tabulated one (t_{tab}). Therefore, a total average recovery of both levels could be considered when t_{exp} was lower than t_{tab} (Bendicho, Martí, Hernández, & Martín, 2002).

3. Results and discussion

MBTH method is so far the most sensitive method for the reducing sugar measurements. However, this method is rarely reported for the hydrolytic activity assay. Therefore, a series of experiments were performed to characterize and optimize the procedure of the lysozyme activity assay.

3.1. Selection of detection wavelength

MBTH has been frequently utilized for the spectrophotometric determination of aldehydes, indoles, aromatic amines, iminohetero-aromatic compounds, arylalkylamines, carbazoles, phenols, reducing sugars, etc. Very different wavelengths were used to monitor the colored products (Anthon & Barrett, 2002; Ribeiro, Prior, Santos, Lopes, & Lima, 2009). MBTH was found to react with lysozyme hydrolyte of half deacetylated-chitosan and N-acetylglucosamine, in the presence of ammonium ferric sulfate to produce a blue colored derivative with maximum absorption at 615 nm and 655 nm. Their spectra against reagent blank and reagent blank against distilled water are shown in Fig. 1. It should be pointed out that the reagent blank had non-negligible absorption at these wavelengths especially at around 615 nm. To further study the detection wavelength, three repetitions of ten different concentrations of N-acetylglucosamine were performed for the standard curve displayed in its slope and R^2 at each nm of wavelength, as shown in Fig. 2. The results revealed that measurement of low levels of N-acetylglucosamine at 655 nm has achieved higher precision (lower RSD) and better linearity (R^2) than that at 615 nm, although partially losing its sensitivity (shown as slope of the calibration curve). Therefore, 655 nm was selected for the RSE and N-acetylglucosamine assay, which was less consistent with Anthon and Barrett's method (2002).

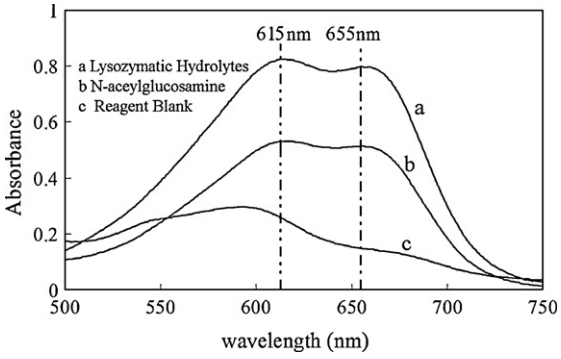

Fig. 1. Absorption spectra of the colored products of MBTH method. The figure shows the absorbance of the products that produced by MBTH reagent reacted with (a) N-acetylglucusamine and (b) lysozyme hydrolytes of half-deacetylated chitosan, respectively versus reagent blank; and reacted with (c) reagent blank (versus distilled water).

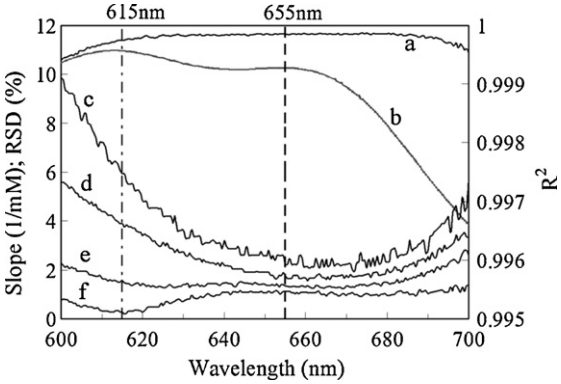

Fig. 2. Effect of detection wavelength on the measurement of N-acetylglucosamine. The figure shows the R^2 (a) and slope (b) of calibration line (0–20 μg mL^{-1}) and relative standard deviation (RSD) of N-acetylglucosamine aliquotes (n = 3) at concentrations of 3.75 μg mL^{-1} (c), 7.5 μg mL^{-1} (d), 10 μg mL^{-1} and (e), 15 μg mL^{-1} (f).

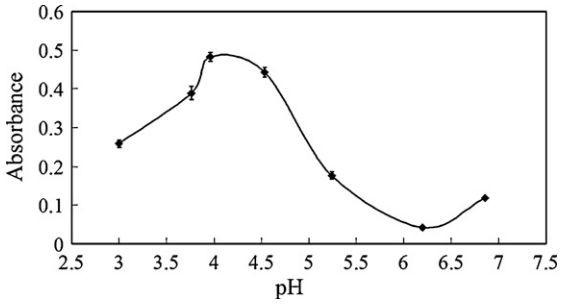

Fig. 3. Effects of pH on the chitinolytic activities of lysozyme (40 °C, 4 mg mL^{-1} chitosan with 52.4% degree of deacetylation, measured at 655 nm).

3.2. Effect of the degree of deacetylation of chitosan on the activity assay

Half-deacetylated chitosan offers three advantages in the activity assay. Firstly, it has a good solubility in aqueous solution within almost all pH range to facilitate the inactivation of the enzyme with NaOH, and avoid precipitation when redox reaction of the products with MBTH happened in alkaline medium. Secondly, it has better susceptibility to lysozyme. The susceptibility of high-deacetylated chitosan to lysozyme is relatively low. We can hardly quantify the production of additional reducing ends with regularly used method, and the initial velocity cannot be measured accurately. Therefore, half-deacetylated chitosan is used in this paper to overcome this problem, which is very important according to our results in this paper. Thirdly, it has high molecular weight. O-hydroxyethylated chitin, carboxymethyl-chitin, dihydroxypropyl-chitin dye-labled chitosan was reported to substrates of lysozyme. Since they were all derived from low-molecular weight of chitosan, the substrates themselves comprise a high proportion of reducing ends which may interfere with the measurement.

3.3. Effect of pH on the activity assay

The effect of pH was studied at different pH values in 50 mM Na-acetate buffer, using half-deacetylated chitosan as substrates (Fig. 3). It is observed that the optimum near pH 4 is in agreement with the results reported by Nordtveit and coworkers using the viscosity method. These results differ from the turbidimetric method (Gorin et al., 1971), in which the optimum is at pH 6.0–6.5. This is presumably due to the complex components of the bacterial cell wall.

3.4. Effect of temperature on the activity assay

Chitinolytic activities of lysozyme were also measured at different temperatures in the range of 30–90 °C. As shown in the inset of Fig. 4, the activity gradually increased with the temperature increasing and reached a maximum at 80 °C, and then decreased sharply at 90 °C. Considering that the relatively high viscosity of the substrate at low temperature (30 °C) predominantly influenced the mass transfer of the enzymatic reaction, and that non-enzymatic hydrolysis and Maillard reaction (Zeng et al., 2007) at relatively high temperature would provide more blank designs in case of the intercepts of the product progress lines on the vertical axis increased with increasing the temperature and more depart from the origin, according to Fig. 4, we pick 40 °C as the testing temperature. As Fig. 5 showed, inactivation of lysozyme during the hydrolysis was negligible, and the product time course remained linear within 1 h. Therefore, this facilitates the most convenient measurement of initial velocity on a single time point or end point in test tubes.

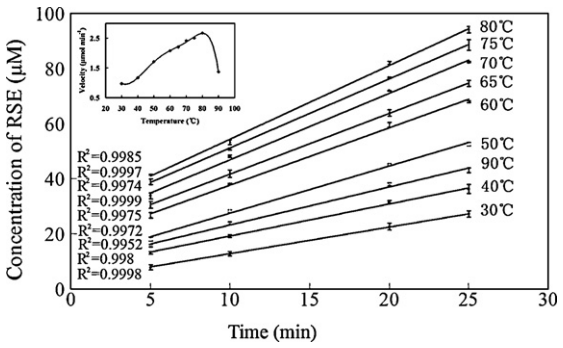

Fig. 4. Effects of temperature on the chitinolytic activities of lysozyme. The inset is to show the activities as a function of temperature (°C). The assay parameters are pH 4.0, with the final concentration of 4 mg mL^{-1} chitosan with 52.4% degree of deacetylation, measured at 655 nm. The concentration of reducing sugar ends (RSE) is calculated according to the calibration line of N-acetylglucosamine.

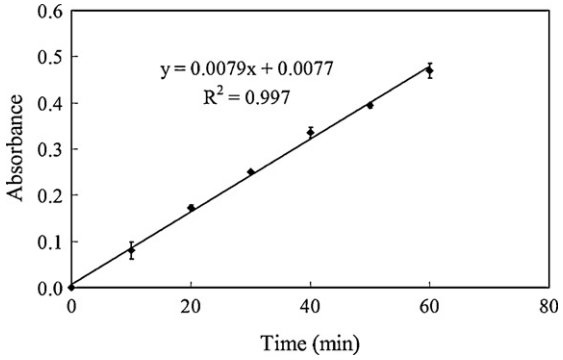

Fig. 5. Product progress curve for chitinolytic activity assay of lysozyme at 40 °C, pH 4.0, with the final concentraion of 4 mg mL^{-1} chitosan with 52.4% degree of deacetylation and 100 U mL^{-1} lysozyme, hydrolyzed for 60 min, measured at 655 nm. To offset the changes of chitosan itself during assay, the same procedure was made without adding the enzyme.

3.5. Effect of substrate concentrations on the activity assay

To further optimize the method, the dependence of the reaction velocity on the concentration of the substrate half-deacetylated chitosan was studied. Fig. 6 shows lysozyme activity as a function of the concentration of half-deacetylated chitosan from 0.625 to 6 mg mL^{-1}. The reaction rate was seen to obey the Michaelis–Menten equation. According to the Lineweaver–Burk plot in the inset of Fig. 6, the kinetic parameter, K_m was at 2.81 ± 0.27 mg mL^{-1}. The velocity approaches a plateau asymptotically as the substrate concentration increases. However, the high viscosity associated with high concentration of half-deacetylated

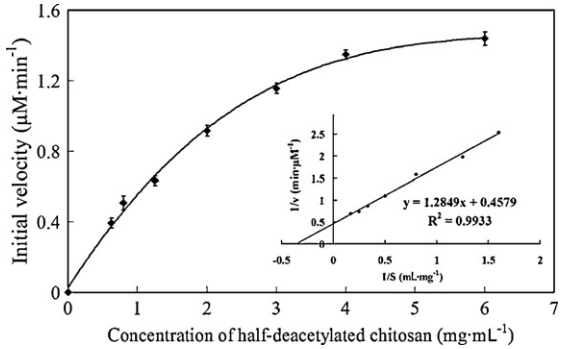

Fig. 6. Michaelis–Menten plot and Lineweaver–Burk plot (inset) of lysozyme. The measurement conditions are as Fig. 5 with the substrate concentration varied.

Table 1
The recovery of the assayed method for the determination of lysozyme activity.

Recovery[a]		Cochran test[b]	Student test-100[c]	Total average recovery	Student test-L[d]
Level 1	Level 2				
97.5 ± 4.6	94.3 ± 6.2	0.640	1.004	95.9 ± 5.5	1.838

[a] Standard addition procedure using two addition levels (46 and 92 U lysozyme mL^{-1}) based on the lysozyme sample containing 30.6 U mL^{-1}, six replicates in each level.
[b] Cochran's test, $C_{tab}(2, 5, 0.05) = 0.877$.
[c] Test to determine differences among the mean recovery obtained and the theoretical 100%, $t_{tab}(11, 0.001) = 4.437$.
[d] Test to determine differences on the recoveries obtained in levels 1 and 2, $t_{tab}(10, 0.001) = 4.587$.

chitosan brings further measurement errors due to the impact of the mass transfer of the reaction. Consequently substrate concentration of half-deacetylated chitosan was selected as 4 mg mL^{-1}. Under such conditions, no more than 0.5% of its reducing sugar ends were released within 1 h of the reaction time, and the reaction velocity at this level decreased slightly with the substrate substrate consumption. Therefore, the product progress course remained appropriately constant, although it possibly followed first-order reaction. For these circumstances, other low-sensitive methods as DNS method, Somogyi–Nelson's method, ferricyanide method, cannot be used in this assay.

3.6. Method validation

The reliability of this method was validated through their linearity, sensitivity, precision, and recovery.

Absorbance responses to the chitinolytic activity of 0–122 U mL^{-1} of lysozyme were linear, and the equation of calibration line was $y = 0.00482x + 0.0067$, as shown in Fig. 7. The relationship of the two methods, turbidimetric method (x, U mL^{-1}) and MBTH method (y, nM min^{-1}), was $y = 8.196x + 5.698$, and the correlation coefficient (R^2) is 0.9975. The values of DL and QL were 3.2 and 10.8 U mL^{-1}. Therefore, the quantitative linear range of the activity was calculated as 94–1006 nM min^{-1}. Six measurements were performed for samples containing 76.5 U mL^{-1} (5 μg mL^{-1}) of the lysozyme, and the relative standard deviation (RSD) of its lysozyme activity was obtained as 2.76%, lower than $RSD_{Horwitz}$ (8.41%). Therefore, the RSD value is acceptable.

The data of Table 1 shows that the homogeneity of variances of the recovery was verified through a Cochran test ($C_{exp} < C_{tab}$). The Student test showed that the recovery of the lysozyme activity did not depend on the enzyme concentration (t-test-100: $t_{exp} < t_{tab}$). Therefore, the final recovery was the average of the results obtained in both levels of addition for each product. Moreover, all the products achieve a recovery appropriate to the theoretical 100% (t-test-L: $t_{exp} < t_{tab}$).

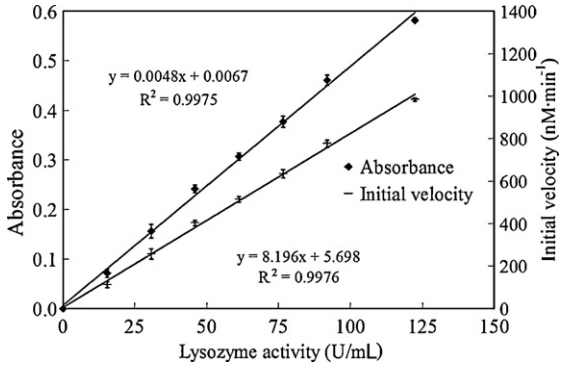

Fig. 7. Lysozyme activity curve with respect to absorbance and initial velocity. The unit of lysozyme is calculated using turbidimetric method. The measurement conditions are as Fig. 5 with the lysozyme final concentration varied, after 1 h of hydrolysis.

4. Conclusions

In this paper, we described a method for quantitative determination of chitinolytic activities of lysozyme, based on the catalytic hydrolysis of half-deacetylated chitosan. The method is simple, rapid, and inexpensive to perform. The optimal procedures are: the pre-warmed half-deacetylated chitosan and lysozyme were mixed to make the final solution containing 4 mg mL^{-1} half-deacetylated chitosan and 94–1006 nM min^{-1} (10.8–122 U mL^{-1}, turbidimetric method) lysozyme at pH 4.0 in sodium acetate buffer, and incubated at 40 °C for 1 h within steady state time course. The reducing sugar ends were measured with our modified MBTH method. Data show that our method is accurate, reliable and precise, and can be also used for accurate determination of specific activities and kinetic parameters of chitinase and chitosanase.

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