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A rapid and accurate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay for quantification of bacteriocins with nisin as an example*

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Abstract: The objective of this study is to propose a more accurate and faster MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay (MCA) for quantitative measurement of polypeptide bacteriocins in solutions with nisin as an example. After an initial incubation of nisin and indicator bacterium *Micrococcus luteus* NCIB 8166 in tubes, MTT was added for another incubation period. After that, nisin was quantified by estimating the number of viable bacteria based on measuring the amount of purple formazan produced by cleavage of yellow tetrazolium salt MTT. Then MCA was compared to a standard agar diffusion assay (ADA). The results suggested a high correlation coefficient (r^2=0.975±0.004) between optical density (OD) and the inhibitory effect of nisin on a bacterial strain *Micrococcus luteus* NCIB 8166 at a range of 0.125~32 IU/ml. The MCA described in this study was very quick. Quantification of nisin took only 7~8 h and the detection limit was at the level of 0.125 IU/ml when compared to 12 IU/ml and 24~28 h for ADA. The MCA provides an accurate and rapid method for quantification of nisin in solutions and is expected to be used for quantification of other antimicrobial substances.

Key words: MTT colorimetric assay, Nisin, *Micrococcus luteus*
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INTRODUCTION

Bacteriocins produced by lactic acid bacteria are natural antimicrobial peptides or proteins with potential applications in food preservation and health care. Nisin is the best-known bacteriocin, and has been used as a safe food preservative (Delves-Broughton, 1990; Deegan *et al*., 2006) to maintain healthy teeth and treatment of gastric ulcer (Howell *et al*., 1993; Reddy *et al*., 2004). A major difficulty of nisin in research and applications is to obtain correct quantification. Therefore, there have been many techniques proposed for detection of nisin. Generally, they fall into the following categories: (1) methods based on bacterial inhibitory effect, such as tube dilution (Parente *et al*., 1995), turbidity assay (Turcotte *et al*., 2004) and agar diffusion assay (ADA) (Tramer and Fowler, 1964; Wolf and Gibbons, 1996); (2) methods based on the immunological principles by using polyclonal or monoclonal antibodies, for examples, sandwich-type enzyme-linked immunosorbent assays (ELISA) (Falahee *et al*., 1990), competitive direct and indirect ELISA (Suárez *et al*., 1996a; Daoudi *et al*., 2001) and dot immunoblot assay (Bouksaim *et al*., 1998); (3) bioluminescence assay (Wahlström and Saris, 1999; Immonen and Karp, 2007), where nisin is quantified by detecting the activity of luciferase in an indicator bacterium *Lactococcus lactis* containing nisin inducible promoters connected to genes of luciferase operon.

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Recently, some new methods were developed for more accurate quantification of nisin and other lactic acid bacteria bacteriocins. These methods are (1) ATP (adenosine triphosphate)-bioluminometry (Valat *et al*., 2003), (2) GFP (green fluorescent protein)-bioassay (Wahlström and Saris, 1999; Reunanen and Saris, 2003; Hakovirta *et al*., 2006), (3) capillary zonal electrophoresis (Rossano *et al*., 1998), flow cytometry (Budde and Rasch, 2001), (4) conductivity measurement (Giraffa *et al*., 1990), automated turbidometry (Skyttä and Mattila-Sandholm, 1991) and (5) matrix-assisted laser desorption (Rose *et al*., 1999). However these methods need delicate equipments and supplies and have not gained wide acceptance. The ADA is still the most widely used quantitative technique for detection of bacteriocins (Wolf and Gibbons, 1996; Wahlström and Saris, 1999; Pongtharangkul and Demirci, 2004). But the major deficiency for ADA is that it is time consuming and laborious. In this study, a rapid, accurate and precise MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay (MCA) is proposed for quantification of bacteriocins using nisin as an example by estimating the number of viable bacteria after incubation with nisin. The viable bacteria are quantified by measuring the cleavage of the yellow tetrazolium salt MTT into purple formazan in the presence of metabolically active bacterial cells.

MATERIALS AND METHODS

Indicator bacterial strain and cultivation

Micrococcus luteus NCIB 8166 was provided by Zhejiang Silver-Elephant Bioengineering Co., Ltd., China, and used as an indicator strain. Culture medium S_1 broth was composed of 0.8% tryptone (Sinopharm Chemical Reagent Co., Ltd., China), 0.5% yeast extract (Hangzhou Tianhe Microorganism Reagent Co., Ltd., China), 0.5% D-glucose (Sinopharm Chemical Reagent Co., Ltd., China), 0.5% NaCl and 0.2% Na_2HPO_4. The reagents were dissolved in distilled water, and sterilized in autoclave at 121 °C for 20 min. A loop of *Micrococcus luteus* was inoculated on the S_1 agar (1.6%, w/v) plate and incubated at 37 °C for 18~24 h. Then, a single colony of bacteria was transferred from the S_1 agar to S_1 broth and incubated at 37 °C for 12 h. Next, bacteria culture was adjusted using the McFarland standard and calibrated to a working concentration of 10^6 CFU/ml in fresh S_1 broth. Finally it was diluted in 10-fold to 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/ml.

Optimization of indicator strain concentration and incubation time with MTT dye

MTT (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate buffered saline (pH 7.2) to a concentration of 5 mg/ml, and then filtered through a 0.2-μm syringe filter. One hundred microlitres of MTT solution was added into each of 2 ml fresh S_1 broth with indicator bacteria (from 10^6 to 10^1 CFU/ml) and then incubated at 37 °C for 1, 2, 3, 4, 5 and 6 h, respectively. When the incubation was finished, the broth culture was kept in boiling water for 5 min to stop reaction. After cooling, the cultures were centrifuged at 1500×g for 20 min to precipitate formazan crystals and the supernatant was removed. To dissolve the formazan crystals, 2 ml of dimethylsulfoxide (DMSO) was added and then the mixture was incubated for 10 min at room temperature. The optical density (OD) of the formazan solution was measured at the wavelength of 510 nm.

Determination of incubation time of nisin with indicator bacteria

1. Preparation of nisin standard

Nisin (Sigma Chemical Co., St. Louis, MO, USA) was suspended in 0.02 mol/L HCl to a final concentration of 1024 IU/ml as a standard solution.

2. Optimization of incubation time of nisin with indicator bacteria

Nisin standard solution was diluted in 2-fold with S_1 broth into tubes containing nisin from 2^9 to 2^{-4} IU/ml. Four tubes (in Groups A, B, C and D) were made for each concentration of nisin. Indicator bacterial suspension was then added to form a final concentration of 10^5 CFU/ml which was optimal as determined in the above experiment. As soon as the tubes in Groups A, B, C and D were incubated at 37 °C for 0, 2, 4 and 6 h, respectively, 100 μl of MTT dye was added for another 2 h incubation. When the incubation was finished, the tubes were kept in boiling water for 5 min to stop reaction. After cooling, the cultures were centrifuged at 1500×g for 20 min to precipitate formazan crystals and the supernatant was removed. The methods for dissolving formazan and

determining the OD value of formazan solution followed the same procedure.

Quantification of nisin and recovery rate in test samples

Standard solution of nisin (1024 IU/ml) described in the preparation section of this report was diluted in 0.02 mol/L HCl to 20, 40, 80, 160 and 320 IU/ml. The tubes containing nisin and bacteria (10^5 CFU/ml) were incubated for 4 h, and then 100 μl of MTT dye were added for another 2 h incubation. The procedures for dissolving formazan and determining OD value of formazan solution were the same as previously described. From the standard solution reading, a regression equation was obtained for OD as an exponential function of nisin concentration. Recovery rate was obtained by dividing the mean of measured values from the actual values, and then multiplying by 100% (Hu, 2005).

Agar diffusion assay (ADA)

We used the ADA as a reference in the detection of nisin activity. The procedure was similar to that as described by Pongtharangkul and Demirci (2004). Briefly, S_1 agar containing 1.5% (w/v) Tween 20 was autoclaved and cooled to 45 °C. An overnighted culture of *M. luteus* was then added in a final concentration of 0.2% (v/v). Precisely 210 ml of this suspension was poured into each sterile Petri plate (280 mm×210 mm). After that, plates were kept at room temperature for 2 h to allow agar solidification. The plates were then stored at 4 °C for 24 h. Holes were bored on the plates using a 7-mm outer diameter stainless steel borer. One hundred microlitres of nisin standard solution (20, 40, 80, 160 and 320 IU/ml) as a test solution was dispensed to each well. The plates were then incubated at 30 °C for a minimum of 20 h to give a well-defined inhibition zone. Seven wells were used for each nisin concentration. Using a caliper, inhibition zone diameters were measured to the nearest 0.1 mm. The mean of the largest and shortest diameters was calculated for oval inhibition zones. A regression equation was calculated for inhibition zone diameter as a function of logarithmic nisin concentration.

Statistical analysis

Data on the activity of nisin were $\log_2$-transformed, but those on the concentration of bacteria were $\log_{10}$-transformed. The correlation between the activity of nisin and the OD_{510} value was analyzed with linear regression using Microsoft Excel 2003.

RESULTS

Optimization of indicator bacteria concentration and incubation time using MTT dye

The production of formazan (OD_{510}) was found directly proportional to both logarithmic concentration of bacteria and incubation time with MTT. Generally, the OD values increased slowly when the concentration of bacteria was higher than 10^5 CFU/ml. On the curve for 2 h incubation, the OD value and logarithmic concentration of bacteria had a higher linear correlation when the concentration of bacteria was between 10^4 and 10^6 CFU/ml. Therefore, the median 10^5 CFU/ml was used in our test system. A 2-h period was used for the incubation of indicator bacteria with MTT in our later experiments because it was a shorter incubation period.

Optimization of incubation time of indicator bacteria with nisin

When the incubation time of nisin with indicator bacteria was increased, so did the production of formazan, but the change of formazan production became less when the incubation time was over 4 h. Therefore, 4 h were used in our later experiment. Although there was a change of formazan production for 4 h incubation when the amount of nisin was less than 2^{-3} (0.125) IU/ml and over 2^6 (64) IU/ml, a better linear correlation of the OD value and the concentration of nisin was found when the nisin was between 0.125 and 32 IU/ml, indicating that the best detection range was between 0.125 and 32 IU/ml.

Regression equation

A high correlation coefficient (r^2=0.975±0.004) was obtained when the linear regression was analysed for the relation between the quantities of formazan expressed as OD values and the activities of nisin at the range of 0.25~32 IU/ml.

Quantification of nisin and measurement of recovery rate in samples

This study measured nisin solutions with con-

centrations known as 20, 40, 80, 160 and 320 IU/ml, respectively, by the MCA proposed. The mean recovery rate was 99.05%, and relative standard deviation (RSD) was 1.56%.

Comparison of MTT colorimetric assay (MCA) and agar diffusion assay (ADA)

A high linear correlation (r^2=0.999±0.004) was found between the ADA and MCA after analysis of the data collected from quantification of 5 nisin samples with the two methods.

Table 1 shows a comparison of MCA and ADA. The highest error in ADA was 6.8% while it was only 5.7% in MCA. As the results suggest, the accuracy is higher using MCA and the time is much shorter than the ADA.

Table 1 Determination of nisin by MTT colorimetric assay (MCA) and agar diffusion assay (ADA) (IU/ml)

Nisin in test samples	Measured by ADA			Measured by MCA		
	Mean	*SD*	Error (%)	Mean	*SD*	Error (%)
20	19.08	1.14	4.6	18.86	0.74	5.7
40	39.40	1.84	1.5	38.58	0.43	3.6
80	74.59	1.64	6.8	81.47	0.86	1.8
160	156.60	10.71	2.1	162.10	1.39	1.3
320	323.21	24.01	1.0	324.21	2.64	1.3

SD: Standard deviation; Error (%)=(|mean−expected value|/expected value)×100

DISCUSSION

There are two primary methods for quantification of bacteriocin activity: bioassays and immunological methods. Bioassays have been the most common methods to measure bacteriocins. These methods are usually semi-quantitative and carried out by serial two-fold dilutions. In these assays, the titre is generally expressed as the reciprocal of the highest dilution that inhibits growth of the indicator organism under the test conditions. Therefore, the semi-quantitative method is inaccurate and time-consuming. Inaccuracy mainly results from the discontinuous scale of two-fold dilutions of the sample. And small differences in bacteriocin concentration cannot be detected by this method, while the immunological methods have high sensitivity. For instance, an ELISA-dependent assay could detect pure nisin or a commercial nisin preparation in cheese at a concentration as low as 0.5 and 200 ng/ml, respectively (Falahee *et al*., 1990); competitive direct ELISA assays using polyclonal and monoclonal antibodies could detect nisin at a concentration of 5~10 and 10 ng/ml, respectively (Suárez *et al*., 1996b); and a dot immunoblot assay had a detection limit at 3 ng/ml for pure nisin or 155 ng/ml for nisin in milk and whey (Bouksaim *et al*., 1998). However, results from these methods are suspicious as the antibodies utilized in these assays sometimes had cross-reactions with related compounds (Bouksaim *et al*., 1999). Moreover, the degraded bacteriocin without antibacterial activity may contribute to the results obtained by the ordinary immunological assay.

In another study, Wahlström and Saris (1999) described a more sophisticated bioassay. In this assay, plasmid pLEB535 with a nisin inducible promoter connected to luciferase gene was transferred into *Lactococcus lactis* (this strain does not produce nisin) as an indicator. Nisin is then quantified by detecting the activity of bacterial luciferase. In this study, nisin could be measured to a concentration as low as 0.0125 ng/ml. However, there were two main limitations in this technique (Reunanen and Saris, 2003). One is that the indicator bacteria have to be in the early log phase of growth in order to achieve a larger signal to noise ratio; the other is that the method needs substrate NDA (*n*-decyl-aldehyde) of luciferase, which makes the procedure more complicated. This problem was solved by using the whole luciferase operon connected to the nisin inducible promoter resulting in a method with the highest nisin sensitivity described by Immonen and Karp (2007). However, this method is not recommendable for food as a substantial amount of the spiked nisin could not be detected. The second most sensitive method (Hakovirta *et al*., 2006) was better for nisin quantification as it could detect all of the spiked nisin from different food products.

In the present study, we overcame the need of

sophisticated instruments in above-described methods. Our results revealed that the MCA proposed in this study took less time, had lower detection limit and higher accuracy, and was easier to carry out than the conventional ADA. Since MCA requires only some common equipment, it can be widely applied in farm dairies and other institutes.

Up to now, the ADA is still the preferred method for bacteriocin quantification, particularly for samples having dregs and sediments (Laridi *et al*., 2003). Taking this into consideration, we chose the ADA as a reference method for comparison with the new method described in this paper. The results obtained by ADA and MCA were highly consistent (r^2=0.999).

Moreover, Mosmann (1983) proposed a new technique to measure cellular metabolism and proliferation. It is based on cleavage of the yellow tetrazolium salt (MTT) into purple formazan in the presence of viable cells. These new findings changed the procedure. From then on, MCA has been applied in a wide range of research fields because of its simplicity, rapidity and accuracy, and especially, being free of isotope. Examples include measuring cell sensitivity to chemicals, reaction of immune cells to irritant, activity of cytotoxin and cytokine, and minimal inhibitory concentration (MIC) of anti-epiphyte medicine. MCA was successfully applied to quantify the inhibitory activity of nisin in the present study by using the same mechanism as described by Mosmann (1983). This study optimized the conditions of MCA for nisin quantification. The number of viable bacteria varied due to the inhibitory effect of nisin after an incubation of nisin with indicator bacteria for 4 h. Two hours were taken for the incubation of bacteria and MTT with a final concentration of 0.125 IU/ml to allow sufficient transformation of MTT into formazan, which conforms with the findings of Saravanan *et al*.(2003). Compared to turbidometric microplate bioassay, our results were more reliable as the results from a turbidometric assay are influenced by some dead bacterial cells in the system.

In conclusion, the MCA described in this study is a method permitting the exact quantification of active bacteriocins. It offers several advantages over the existing methods in terms of precision, simplicity, rapidity and accuracy.

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