Novel Method for the Quantitative Analysis of Protease Activity: The Casein Plate Method and Its Applications

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ABSTRACT: No simple methods are used for the quantitative analysis of the protease activity in colored food up till now. Thus, this study aims to establish a new and simple method for the quantitative detection of protease activity, especially in colored food. The detection accuracy, detection limit, and repeatability of the casein plate method were analyzed. Then, the application of the casein plate method in sample detection and recovery was further evaluated. The results showed that the casein plate method for the quantitative detection of protease activity has high accuracy, high precision, and low detection limit. The recoveries of eight kinds of colored samples were in the range of 92.26−97.84%, and the relative standard deviation (RSD) was in the range of 3.56−10.88%. The results of the casein plate method exhibited high accuracy. This indicated that the method was suitable for the detection of colored samples. The casein plate method for the quantitative detection of protease activity is simple. The newly constructed casein plate method has broad potential application value in food industry, especially for the detection of dark food.

1. INTRODUCTION

Proteinase is a kind of enzyme for protein hydrolysis. It is a group of large and complex enzymes with highly specific protein hydrolysis. It features high specificity to biological molecules and has wide application value in the fields of food, medicine, and detergents.1 They are involved in the selective proteolysis of various specific substrates, embryonic development, bone and organ tissue repair, neuron growth, immune and inflammatory cell regulation, angiogenesis and apoptosis, and other biological processes.2−5

The analysis of protease activity is an important step in the research and application of proteinase6 development and validation of a simple titration method for studying the heat-activated; endogenous protease in arrow tooth flounder fillet mince is described. Protease activity has been detected by the fluorescence method,7,8 but detection based on calorimetry,9 mass spectrometry (MS),10 immunoassay,11 electrophoresis,12,13 amperometry,14 optics,15,16 and so on.

Fluorescence-conjugated polyelectrolytes, suspended ionic sulfonates, and carbonyl groups are used to detect protease activity.7 The fluorescence method exhibits an obvious mechanism and entails a simple operation. However, given the need for special instruments, certain operational requirements must be met by experimenters; hence, this method is not commonly employed. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has the advantages of simplicity, high throughput, and sensitivity and thus possesses great application potential. However, this method is not commonly applied because of its high instrument and operational requirements. D1 protease activity has been determined by capillary zone electrophoresis,12 but this method cannot detect the activities of other proteases. Kilian et al.17 proposed an organic derivatization method using silicon-based nanoporous photonic crystals to immobilize polypeptides for the detection of proteases in solutions. The minimum detection concentration of this method is 3.7 × 10−10 mol·L−1. Although this method has a low detection limit and yields accurate detection results, it requires specific nanomaterials, which are usually expensive; hence, it is also not commonly used.

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Mahmoud et al.\textsuperscript{18} proposed a method for the electrochemical detection of human immunodeficiency virus type I protease by surface modification with ferrocene (Fc) peptide with a detection limit of $8 \times 10^{-11}$ mol L$^{-1}$. This method also has a low detection limit and achieves accurate detection but requires electrodes, nanomaterials, and specific electrochemical workstations. It also entails high operational requirements from the experimenters. Serim et al.\textsuperscript{19} described the use of probe technology to detect protease activity. At present, the colorimetric method is commonly used to detect protease activity. Common protease activities are quantitatively expressed by detecting either the decrease in substrate protein concentration or the increase in product-free amino acid or polypeptide concentration before and after enzymatic hydrolysis. Ultraviolet spectrophotometer and biuret reagent are used in the former, while ninhydrin colorimetry, trinitrobenzene sulfonic acid, and Folin-phenol reagent are used in the latter. In the determination of protease activity using an ultraviolet spectrophotometer,\textsuperscript{20} the protease hydrolyzes the casein substrate at a certain temperature and pH, the enzyme reaction is terminated by the added trichloroacetic acid, and the unhydrolyzed casein is precipitated. The activity of the casein can be determined by ultraviolet spectrophotometry. However, this method suffers from disadvantages such as unsuitability for colored sample detection, difficulty of operation, and proneness to errors.

The Coomassie Brilliant Blue method\textsuperscript{21} is also used to detect protease activity. In this method, the Coomassie Brilliant Blue dye binds to the protein under acidic conditions. The dye binds to basic amino acids (especially arginine) and aromatic amino acid residues in protein so that the maximum absorption peak of the dye increases from 465 to 595 nm and the color of the solution changes from brown to black. Absorption is determined at 595 nm. This method is fast, simple, stable, and highly sensitive, but its linear relationship becomes increasingly deviated with the increase in protein content. The reaction can be disturbed by a strong alkaline buffer, sodium dodecyl sulfate, and other substances.\textsuperscript{22} In the ninhydrin chromogenic method,\textsuperscript{23–25} protease activity can be determined by adding protease to the solution containing protease to decompose for a period of time; the content of the produced $\alpha$-amino acid is then determined. However, the results of this method are quite erroneous because of the slow reaction, side reactions, unstable hydrolysis, oxidation and photolysis, and color interference.\textsuperscript{26}

However, there is no simple method suitable for detecting protease activity in dark samples. This is mainly because the commonly used spectrophotometry is seriously disturbed by color.

When the protease activity in real samples is measured, many samples are dark in color, and the extracted enzyme solution is even darker in color. For example, when a sample’s color is extremely dark, it presents a blue-black color after a reaction. For light-colored samples, the complete color of the reaction is dark blue or light blue. Hence, a new method is needed to eliminate the interference of color and accurately determine the enzyme activity of dark samples. The issue is whether a simple way may be used to achieve high accuracy and precise analysis that is unaffected by the sample color?

In the present work, we aimed to establish a new and simple method for the quantitative detection of protease activity, especially in colored samples. Commercial trypsin was diluted and cultured in different solutions. The measured results were used as standard curves. The activity of the samples was calculated according to the size of the hydrolysis circle of the samples. The feasibility of using the casein plate method in the quantitative analysis of protease activity was discussed.

2. RESULTS AND DISCUSSION

2.1. Standard Curve of Casein Plate Method and Limit of Detection (LOD).

The relation between the area of the hydrolysis circle and the activity of commercial trypsin is shown in Figure 1. The regression equation was

$$Y = 1.7289X + 15.668$$

Figure 1. Standard curve for the determination of enzyme activity by the casein plate method.

$R^2 = 0.9986$, $Sa$ (standard deviation of the response) = 2.299, in which $X$ is the activity of commercial trypsin (U mL$^{-1}$) and $Y$ is the area of the hydrolysis circle (mm$^2$). It indicated a good linear relationship between the area of the hydrolysis zone and the protease activity in a wide range.

Moreover, according to eq 1, LOD\textsuperscript{27} could be obtained as follows: $LOD = 3Sa/b = 3.989$ (U mL$^{-1}$), where $b$ is the slope of the calibration curve (eq 1). Jannat et al.\textsuperscript{28} reported a semiquantitative liquid crystal (LC)-based method for the detection of protease with a detection limit of 10 ng mL$^{-1}$ protease (active 7.23 U mg$^{-1}$ protease) or 6.5 $\mu$g mL$^{-1}$ peptide fragment. However, the test results may be affected by undigested casein or residual protease unless the peptide fragment is analyzed by LC. All casein and protease molecules were precipitated by TCA. Mahmoud used the electrochemical method to detect human immunodeficiency virus type I protease, and the detection limit was $8 \times 10^{-11}$ mol L$^{-1}$. The electrochemical method uses an electrochemical workstation to detect protease. The instrument has high precision, low detection limit, and accurate detection results. However, specific and expensive instruments are needed for protease detection. Ingram et al.\textsuperscript{29} constructed a SERRS-based enzyme probe to detect protease activity with a detection limit of 50 ng mL$^{-1}$, which is considerably lower than that of the Folin–Ciocalteu method or the casein plate method. However, this method requires an enzyme probe. Compared with the casein plate method, it has a higher cost, more complex operation, and higher requirements for operators and instruments.
Although the detection limit of the casein plate method is lower than that of the electrochemical and enzyme probe methods, it can meet the general detection accuracy, and the operation is simple and easy.

2.2. Precision. Samples (10 μL, 25, and 50 U·mL⁻¹) were injected into the casein plate. After 10 min of static incubation, the samples were cultured in an incubator at 37 °C for 18 h. The area of the hydrolysis circle was calculated to measure the enzymatic activity. The results are shown in Table 1.

Table 1. Measurement Results and Relative Standard Deviation (RSD) Values of Low and High Concentrations

| sequence number | low concentration (25 U·mL⁻¹) | high concentration (50 U·mL⁻¹) |
|-----------------|-------------------------------|--------------------------------|
|                 | measurement results           | RSD (%)                        | measurement results | RSD (%)                        |
| 1               | 24.449 ± 0.110                | 47.917 ± 0.996                 | 2.08               |
| 2               | 26.348 ± 0.793                | 47.389 ± 1.353                 | 2.85               |
| 3               | 26.0295 ± 1.015               | 46.925 ± 0.806                 | 1.72               |
| 4               | 25.316 ± 1.116                | 46.432 ± 0.567                 | 1.22               |
| 5               | 25.238 ± 1.227                | 48.015 ± 0.621                 | 1.29               |
| 6               | 24.686 ± 0.885                | 47.917 ± 0.996                 | 2.08               |

*RSD indicates relative standard deviation.

The precision results of this method are shown in Table 1. The RSD ranged from 0.45 to 4.86% at low concentration and from 1.22 to 2.85% at high concentration. Wu et al.³⁰ used high-performance liquid chromatography-ultraviolet (HPLC-UV) detection to determine the content of phthalate esters and obtained an RSD range of 3.9—5.7%. The RSD of the casein plate method was 0.45—4.86%, which was similar to HPLC. This finding was mainly due to the fact that the hydrolysis circle of the casein plate was somewhat irregular, resulting in some operational errors in manual measurement. However, the RSD values measured via the casein plate method were all less than 5%, which indicated a relatively good precision.

2.3. Repeatability. Repeated experiments were carried out at a moderate concentration (25 U·mL⁻¹). The results are shown in Table 2.

2.4. Detection of Protease Activity in a Colorless Sample. The casein plate hydrolyzed by the commercial trypsin solution (colorless sample) is shown in Figure 2a,b. The casein plate that reacted with the trypsin solution clearly exhibited a hydrolysis circle with a smooth and clear outline, while the casein plate that was hydrolyzed by sterile water had no hydrolysis circle, indicating the absence of protease activity. The smooth edge of the hydrolysis circle can effectively reduce measurement error and obtain accurate measurement results.

The commercial protease solution of 25 U·mL⁻¹ concentration was determined by the casein plate method. The enzymatic activity of the commercial protease solution by the casein plate method was 24.53 ± 0.66 U·mL⁻¹, with a RSD of 2.7%. Thus, the casein plate method demonstrates good credibility for colorless samples.

2.5. Detection of Protease Activity in Colored Samples. After 24 h of phosphate-buffered saline (PBS) extraction, the samples were filtered using a slow qualitative filter paper. The filtrate was centrifuged for 10 min at 8000 rpm to precipitate the bacteria and obtain a crude enzyme solution. The crude enzyme solution (10 μL) was injected into a perforated casein plate. After 10 min of standing, the casein plate was cultured in an incubator at 37 °C for 18 h. The diameter of the hydrolysis circle was measured, and its enzyme activity was calculated.

The casein plate of the soy sauce sample is shown in Figure 3. It shows that no hydrolysis circle is formed in Figure 3a, indicating that the sample has no protease activity and that the substrate casein cannot be hydrolyzed. Figure 3b shows that the sample hydrolyzed a certain amount of substrate casein at a certain range; hence, an obvious hydrolysis circle is formed. The area of the hydrolysis circle and the protease activity are calculated according to the standard curve of commercial trypsin.

The activity of protease was determined by the casein plate method, the results are shown in Figure 3c. The protease activity of eight kinds of colored foods extracts was determined by the casein plate method. The relative standard deviation of the results was within 4.265—10.35%, which indicated that the error was small and the results were accurate and reliable.

2.6. Analysis of Recovery Rates by Standard Addition to Colored Sample. The recoveries of eight kinds of colored foods by the casein plate method is shown in Table 3. The recovery of the casein plate assay ranged from 92.26 to 97.84%, and the relative standard deviation ranged from 3.56 to 10.88%. According to the Chinese national standard GB/T 2704—2008 “Laboratory quality control specification-physical and chemical inspection of food,” when the content of crude components is less than 0.1 mg·kg⁻¹, the recovery rate of the added standard should be between 60 and 120% and that of the casein plate method is 92.26—97.84%. In this range, the casein plate method determines the protease activity more accurately. So, the casein plate method is especially suitable for measuring the activity of protease in the colored samples.
The casein plate method mainly solves the problem of interference of the dark-colored sample extract in the common methods for detecting protease activity. Moreover, the casein plate method is simple, easy to operate, and appears obvious hydrolysis ring. It can effectively avoid errors caused by color. Therefore, the casein plate method is especially suitable for the detection of protease activity in colored food.

3. CONCLUSIONS

We constructed a novel and simple method for the accurate and precise quantitative analysis of the protease activity-casein plate method. The precision experiment showed that the RSD of the casein plate method was between 1.22 and 8.07%, which indicated good precision. Its detection limit was 3.989 U·mL⁻¹, and its repeatability RSD was 2.1–5.73%; both values indicated good repeatability. The casein plate assay is suitable for the detection of protease activity in colorless samples (such as commercial trypsin solution). Moreover, the casein plate method for evaluating the protease activity of eight colored samples exhibited a low RSD. The recoveries of eight colored samples determined by the casein plate method ranged from 92.26 to 97.84%, and the results were accurate. The advantage of the casein plate method is that it can effectively avoid errors caused by deep-colored samples. It has no requirement for sample color, especially for dark samples, such as red pitaya. It is simple to operate and can be widely used. It can also be effectively applied to determine the enzymatic activity of soy sauce, tea, and other products with darker colors.

4. MATERIALS AND METHODS

4.1. Materials. Trypsin (25 000 U·g⁻¹) was purchased from Biofroxx Co., Ltd., Germany. Analytically pure glucose, potassium dihydrogen phosphate, sodium carbonate, potassium biphosphate, trichloroacetic acid, and magnesium sulfate
were purchased from Chemical Reagents Co., Ltd. of the National Pharmaceutical Group. All other chemicals used in this work were of analytical grade and commercially available. Red heart pitaya, amaranth, and blueberry were purchased from Wal-Mart supermarket in Huaxi district of Guiyang city; soy sauce was obtained from a local soy sauce manufacturer and not sterilized.

### 4.2. Preparation of Casein Plate.

The casein plate medium contained casein 10 g·L⁻¹, glucose 1 g·L⁻¹, yeast extract 1 g·L⁻¹, KH₂PO₄ 0.1 g·L⁻¹, KH₂PO₄ 0.5 g·L⁻¹, magnesium sulfate 0.1 g·L⁻¹, and agar 20 g·L⁻¹. Then, 20 mL of the mixture was injected into a plate with a diameter of 9 mm. To ensure the thickness uniformity of the plate, we use the plates of the same size (90 mm), add the same amount of casein to the plates, and put the plates flat.

### 4.3. Casein Plate Method for a Quantitative Analysis of Protease Activity and Limit of Detection (LOD).

Protease can hydrolyze the casein plate to form an observable hydrolysis circle. The prepared casein plate was drilled with a 10 mL gun head (three parallel holes in one plate), and 10 μL of enzyme solution was injected into the hole. After 10 min, the casein plate was placed in an incubator at 37 °C for 18 h. The diameter of the hydrolysis ring was measured, and the area of the dissolution ring was calculated. Commercial trypsin was plotted with the area of the dissolving circle as the ordinate (μm²) and with the activity of commercial trypsin as the abscissa (X, U·mL⁻¹). Then, the linear regression equation for Y and X could be obtained as follows:

\[
Y = a + bX
\]  

where \(b\) is the slope of the calibration curve. Also, LOD can be detected²⁷ according eq 3

\[
LOD = 3Sa/b
\]  

where \(Sa\) is the standard deviation of the response \(Y\) and \(b\) is the slope of the calibration curve (eq 2). Sample enzymatic activity was determined according to the standard curve. Here, why do we choose the incubation time of 18 h? Our preliminary experiments (data not shown) indicated that when the incubation time of the casein plate is less than 18 h, the hydrolysis circle of samples with low enzyme activity is not obvious, and the error of detecting enzyme activity is large. However, if the time is too long, the rate of change of the hydrolysis circle will decrease rapidly. Therefore, considering the detection effect and saving time, the culture time selected by this method is 18 h.

### 4.4. Precision Experiment.

Eighteen copies of 0.100 g of commercial trypsin were dissolved in sterile water at a constant volume of 10 mL. A 250 U·mL⁻¹ trypsin solution was prepared and diluted into 25 and 50 U·mL⁻¹ solutions. Each casein plate was injected with 10 μL of enzyme solution and incubated at 37 °C for 18 h after 10 min of standing. The diameter of the hydrolysis circle was measured, and the enzyme activity and the relative standard deviation (RSD) values were calculated.

### 4.5. Determination of Enzyme Activity in Colorless Sample.

The commercial trypsin (0.100 g) was accurately weighed and dissolved in sterile water to a constant volume of 10 mL to prepare a 250 U·mL⁻¹ trypsin solution. The casein plate method was used for the determination.

### 4.6. Determination of Enzyme Activity in Colored Samples.

Red pitaya fruit, soy sauce, dried mulberry, purple cabbage, amaranth tricolor, red rice, black rice, and pineapple (both 1 g) were extracted with 10 mL of PBS buffer for 24 h. After that, the extraction solution was filtered with a 0.45 μm porous membrane for the preparation of the enzyme solution. The casein plate was perforated, and 10 μL of the enzyme solution was injected into the hole. After 10 min of standing, the casein plate was cultured at 37 °C for 18 h. The diameter of the hydrolysis ring was measured, and the enzyme activity and the RSD values were calculated.

Then, the extract of colored foods was added with some amount of 10 U·mL⁻¹ of commercial trypsin solution. The mixed liquid (10 μL) was injected into the casein plate pore and incubated at 37 °C for 18 h. Then, the enzyme activity and recovery rate were measured.

### 4.7. Statistical Analysis.

All experiments were performed in three replicates, and the data are presented as the mean ± standard deviation (SD), and their RSD values were calculated.

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**Notes**

The authors declare no competing financial interest.

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