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A high sensitive assay platform based on surface-enhanced Raman scattering for quantification of protease activity

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ABSTRACT

In this study, a new, sensitive, and rapid assay was developed to quantitatively measure the proteolytic enzyme activity using the surface-enhanced Raman scattering (SERS) probe. Two different shapes of gold nanoparticles, gold nanosphere and nanorod particles were produced. SERS label, comprising self-assembled monolayers (SAMs) of Raman reporter molecule (5,5-Dithiobis (2-Nitrobenzoic acid), DTNB), was coated on the surface of the nanoparticles. Two different SERS-based analysis platforms were designed using gold-coated glass slide and polystyrene microtiter plate. The calibration curves were obtained by plotting the intensity of the SERS signal of symmetric NO_2 stretching of DTNB at 1326 cm^{-1} *vs.* the protease concentration. The effects of nanoparticle geometry and assay platform on the protease assay were investigated and the best working combination of the parameters was selected as rod shaped SERS probe and gold-coated glass slide. The correlation between the protease activity and SERS signal was found to be linear within the range of 0.1–2 mU/mL (R^2 = 0.979). The limit of detection (LOD) and limit of quantification (LOQ) values of the validated method were found as 0.43 and 1.30 mU/mL, respectively. The intra-day and inter-day precisions of the method, as relative standard deviation (RSD), were determined as 2.5% and 3.6%, respectively. The developed method was successfully applied for quantitative analysis of the commercial enzyme preparate that is used in cheese making process. It was also used for investigation of substrate specificity of protease enzyme towards the casein and bovine serum albumin. The proposed method has a flexibility to try different substrates for the detection of various enzyme activities.

1. Introduction

Enzymes that are biochemical catalyzers facilitate intra- and extracellular reactions therefore they are required by the living cell. The enzyme which has proteolytic activity, known as protease, is a group of enzyme and it has significant importance in living cell and industrial bioprocess [1,2]. Proteases are also common agents to many viruses and infectious diseases, and have been involved in a host of pathogens [3]. On the other hand, detection of protease activity is very important for the initial step of cancer screening [4]. They are used for medical and pharmaceutical applications [5] and they also have importance in some bioprocesses in the industry. Proteases accounts for 40% of the total worldwide enzyme sales and are one of the most important industrial enzymes group. They are used in different industries for different purposes such as in detergents, leathers and food industries [6–8]. The use of the proteases in food industry is different from the others and they have both desirable and undesirable effects in food processing [9–11]. For example, proteolytic reactions are essential biocatalyzers in cheese making. However, uncontrolled hydrolysis of casein causes cheese defects [11]. In addition, proteases (from exogenous or endogenous origin) can also cause unpleasant flavours and odours in milk [12] and proteases originated from suni bug enzyme in wheat can cause gluten hydrolysis and reduce the bread making capability [13].

Due to the crucial role of protease, it is important to determine the proteolytic activity in both diagnostics and process and quality control. Up to date, several analytical methods have been described to detect and quantify protease activity such as spectrophotometric [6,14,15], electrochemical [16–18], calorimetric [19], chromatographic [20], and immunologic [21]. Such new technologies allow opportunities to develop novel methods which have better analytical properties such as low detection limit, minimum analysis time, low cost, and minimum sample preparation stage.

Surface-enhanced Raman scattering (SERS) is a technique that provides greatly enhanced Raman signal from Raman-active analyte molecules that have been adsorbed onto certain specially prepared metal surfaces. Increases in the intensity of Raman signal have been observed by the factors up to 10^{14} [22]. This remarkable

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enhancement provides high sensitivity and gives an opportunity for the development of ultrasensitive analytical method for chemical and biochemical detection. After the discovery of SERS, it gained much attention and became an interesting technique that is used in different studies such as signal transduction mechanisms in biological and chemical sensing applications [23–25], binding properties of antigen, antibody or DNA strands [26–32], immunoassays [33,34], bacteria or virus [31,35,36] and glucose detection [35]. It is also used for enzyme activity measurement for different enzymes; hydrolases [37], phosphatase [38], peroxidase [39], protease [40] by using specifically designed SERS substrates. It was reported that SERS-based assay technique allows rapid and ultrasensitive analysis of alkaline phosphatase (ALP) at concentrations as low as $\sim 4 \times 10^{-15}$ M using gold nanoparticles as a SERS material whereas 5-bromo-4-chloro-3-indolyl phosphate (BCIP) is used as a substrate of ALP [38]. Label free direct and Raman dye-labelled indirect methods are two major SERS-based detection methods for biomolecule detections [41]. The vibration information of the target molecule is monitored in direct system and target molecule is identified by matching its spectrum [42]. In indirect method, Raman label is used as any other labeling agent and target molecule is quantified [43]. Indirect method is generally enables low levels of detection limits. For example, femtomolar detection of prostate specific antigen in human serum for early disease diagnosis by using DTNB as Raman label was demonstrated by low level LOD [28] Although, direct method gives several advantages such as label free monitoring and minimum analysis period, it becomes difficult to analysis data if the measurement is performed in a real or complex sample matrix.

Despite the fact that there are many studies about quantification of protease activity, studies involving SERS are rather limited. In the present study, a new method was developed for protease activity measurement based on SERS. The aim of the study was to develop a new method that is less time consuming, sensitive, and easy-to-use. For this purpose we used an indirect method by using a SERS labeling agent to measure the protease activity. The parameters that affect the performance of the analysis were investigated and then the method was designed and validation of the method was performed. The method was evaluated in terms of linearity, sensitivity, and precision. Accuracy of the method was tested by quantitative analysis of the commercial enzyme preparate and results were compared with a colorimetric method using azocasein substrate. The substrate specificity of protease enzyme towards the casein and bovine serum albumin was also investigated by the developed analysis platform.

2. Experimental

2.1. Biochemicals and chemicals

Hydrogen tetrachloroaurate ($HAuCl_4$), hexadecyltrimetylammonium bromide (CTAB), L-ascorbic acid (AA), and trisodium citrate dehydrate were obtained from Sigma–Aldrich (Taufkirchen, Germany). N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), 11-mercaptoundecanoic acid (11-MUA), 2-morpholinoethanesulfonic acid monohydrate (MES), protease (Type I: Crude) from bovine pancreas, bovine serum albumin (BSA), casein from bovine milk, sulfanilamide-azocasein (Azocasein), fluorescamine and trichloroacetic acid (TCA; minimum 99.0%) were obtained from Sigma–Aldrich (Steinheim, Germany). Silver nitrate ($AgNO_3$), sodiumborohydride ($NaBH_4$), di-sodium hydrogen phosphate (Na_2HPO_4) hydrogen phosphate solution (30%) and absolute ethanol, were obtained from Merck (Darmstadt, Germany). 5,5-Dithiobis (2-Nitrobenzoic acid) (DTNB) was obtained from Acros (NJ, USA). Potassium dihydrogen phosphate (KH_2PO_4), acetone, and sulfuric acid (95.97%) were purchased from J.T. Baker (Deventer, Holland). N-Hydroxysuccinimide (NHS) was purchased from Pierce Biotechnology (Bonn, Germany). All solutions were prepared with Milli-Q quality water (18 MΩ cm).

2.2. Solutions and buffers

Phosphate buffer solution (PBS; 67 mM, pH 7.5) was prepared by using Na_2HPO_4 and KH_2PO_4. 2-Morpholinoethanesulfonic acid buffer (MES; 0.1 M, pH 6.5) was prepared by adjusting the pH with NaOH. Enzyme solutions (in the concentration range from 0.135 to 1350 mU/mL) were prepared by using PBS. Flourescamine stock solution (1.08 mM) was prepared by using pure acetone.

2.3. Synthesis and characterization of gold nanoparticles

2.3.1. Synthesis of spherical gold nanoparticles

Gold nanoparticles were synthesized using the citrate reduction method according to Sutherland and Winefordner [44]. Briefly, in a 500 mL round-bottom flask equipped with a condenser, 500 mL of 0.01% $HAuCl_4$ solution was prepared and boiled under stirring. Then 7.5 mL of 1% sodium citrate solution was added into boiling solution. After approx. 60 s, the color of the solution changed from pale yellow to color of red wine. Boiling was continued for 15 min to ensure complete reduction. It was cooled down to room temperature in order to make it ready to use.

2.3.2. Synthesis of rod shaped gold nanoparticles

Gold nanorods were prepared by seed mediated growth technique with slight modification [45]. Briefly, seed solution was prepared by mixing 7.5 mL of 0.1 M CTAB solution and 250 μL of 0.01 M $HAuCl_4$ solution and stirred. Thereafter, 600 μL of 0.01 M ice-cold $NaBH_4$ was added all at once and allowed to stay 5 min at room temperature in order to form seed solution. To prepare gold nanorods growth solution, 4.75 mL of 0.1 M CTAB, 50 μL of 0.01 M $HAuCl_4$, and 15 μL of 4×10^{-3} M $AgNO_3$ were mixed, respectively. The color of the resulting was dark orange. Then 15 μL of 0.1 M AA was added drop by drop to the resulting solution in order to get growth solution. The color of solution turned to colorless after adding AA. Then, 2.5 μL seed solution was added to growth solution for fabricating gold nanorods. This final mixture was stirred for a few seconds and allowed to stay 3 h at room temperature for nanorods synthesis.

2.3.3. Characterization of nanoparticles

Absorption spectra for both spherical gold and rod shaped gold nanoparticle solutions were recorded with Agilent 8453 UV–vis spectrophotometer (Agilent technologies, Inc., Santa Clara, CA) with a photodiode array detector. Transmission electron microscopy (TEM) measurements were performed on a JEOL 2100 HRTEM instrument (JEOL Ltd., Tokyo, Japan). TEM samples were prepared by pipetting 10 μL of nanoparticle solution onto TEM grids and allowed to stand for 10 min.

2.4. Raman instrumentation

Delta Nu Examiner Raman Microscopy system (Deltanu Inc., Laramie, WY) equipped with a 785 nm laser source, a motorized microscope stage sample holder, and a cooled charge-coupled device (CCD, cooled to 0 °C) detector was used for protease activity assay. Instrument parameters were 20× objective, 30 μm laser spot size, 30 s acquisition time, and baseline correction was performed for all of the measurements.

2.5. Preparation of extrinsic Raman labels

Equal quantity (1 mg) of spherical gold nanoparticles and gold nanorods were used as Raman labels by assembling DTNB on nanoparticles, separately. For constituting self-assembled monolayer, 50 mM DTNB was used by keeping overnight in absolute ethanol to form the SERS signal on the nanoparticle surfaces. After this procedure, obtained particles were washed and the carboxyl group that is on the free end of the DTNB, was activated by EDC/NHS (0.2/0.05 M in MES buffer for 30 min) for further covalent binding to the substrate which was bound to the support material. After surface activation, nanoparticles were washed with MES buffer and then final solutions were obtained by dispersions of the nanoparticles in 1 mL MES buffer for further usage.

2.6. Assay platform preparation and protease activity measurement

Two different platforms, gold-coated glass slide and polystyrene plate, were designed for protease assay and the performances of the platforms were compared. The schematic illustration of platform design is shown in Fig. 1 and detail was given below. In this stage, effects of the platform design and nanoparticle shape on the assay performance were investigated.

2.6.1. Analysis platform I

Gold-coated glass slides (Aluminosilicate Glass Slides Coated with 500A Au, Platypus Technologies, Madison, WI, USA) were used as support materials that have the substrate on the surface for the enzymatic reaction. Gold-coated slide was cut into proper size (~10 × 10 mm). Slides were cleaned with piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide) and rinsed with water prior to self-assembled monolayer (SAM) formation. In order to bind the substrate to the gold-coated glass slides, SAM was prepared by 75 mM 11-MUA in absolute ethanol by incubating overnight at room temperature. Subsequent to the activation, the slides were rinsed with MES buffer several times and substrates (casein or BSA in phosphate buffer, 2%, w/v) were added to fasten them covalently onto the SAM and incubated overnight at room temperature. At the end of the incubation period, surfaces were rinsed with MES buffer and then interacted with Raman labelled nanoparticles (200 μL) for 1 h by vigorous stirring. Excess of the particles were removed by washing and finally, the analysis platform was ready to use.

The success of the surface activation with EDC/NHS was also investigated by interaction the surface with casein substrate before and after surface activation. After interaction, unbounded casein was removed from surface by washing and then surfaces were reacted with flourescamine to visualize the existent of the casein on the surface. In this experiment, flourescamine reacts with the amino groups of the casein on the surface and fluorescence intensity of the surface increases depend on the amount of the casein on the surface. Fluorescence observations were performed using LEICA DMI4000 B fluorescent microscope with a 100× objective (Leica, Wetzlar, Germany).

2.6.2. Analysis platform II

Polystyrene microtiter plate was used as support material. Substrate (casein or BSA) was adsorbed on the surface of plate well, spontaneously. Prepared support materials and Raman labelled nanoparticles (200 μL) were interacted for 1 h by vigorous stirring. At the end of the interaction, the platforms were washed with MES buffer and finally the platform became ready to use for enzyme activity measurement.

2.6.3. Enzymatic activity measurement

Two different assay procedures were performed using platforms. In the platform I (Fig. 1A), initial spectrum of SERS probe on the platform was determined and then it was immersed in the 200 μL of enzyme solution at different concentrations. It was incubated at room temperature for 30 min to perform enzymatic reaction and then the reaction was ended by washing platform with phosphate buffer for at least three times. The spectrum of remaining SERS probe on the analysis platform was then obtained and reduction of intensity was determined. In the second platform, the reaction was performed in the well of the platform II (Fig. 1B). Enzymatic reaction was started by adding 200 μL of enzyme solution at different concentrations into the well. Reaction was performed at room temperature during 30 min of incubation period. At the end of the incubation period 1 μL of the solution was used for SERS measurement. SERS measurement was performed on the surface of the gold slide and the spectrum of the SERS probe was obtained. Spectral data was further processed with at least 3-point averaging in software to reduce noise and to obtain an average signal of different analysis areas. Enzymatic activity measurements were performed on the platforms composed of spherical and rod shaped SERS probes and calibration graphs, intensity *vs.* enzyme concentration, were obtained. Based on the result obtained at this stage, a proper combination of nanoparticle shape (spherical or rod) and platform (platform I or platform II) were selected. The method, designed based on this combination, was used for the further part of the study. All experiments were performed three times and the average value of three SERS spectra was taken.

Protease activity of the enzyme used in this study was determined by using azocasein method described by Bendicho et al. [14]. Azocasein was used as a chromogenic substrate. The reaction mixture containing 1 mL of 1.0% (w/v) azocasein (prepared in phosphate buffer) and 100 μL of the enzyme sample was mixed and incubated at 25 °C for 30 min. The assay was terminated by addition of 2 mL of 5% TCA. The contents were mixed thoroughly and allowed to stand for 15 min to ensure complete precipitation of the remaining azocasein fragments. The sample was centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was determined at 345 nm wavelength that the maximum absorbance of supernatant was obtained, using UV/Visible spectrophotometer. One unit (U) of protease activity on azocasein was defined as the amount of the enzyme required to produce an absorbance change of 1.0 at 345 nm/min at 25 °C and pH 7.5.

2.7. Validation of developed method

Validation of the proposed method was performed with respect to linearity, sensitivity (limit of detection (LOD) and limit of quantification (LOQ)), precision (intra- and inter-day repeatability), and accuracy. These validation criteria were studied under the optimal analysis condition.

The relation between the protease concentrations (from 0.135 to 1350 mU/mL) and the band intensity at 1326 cm^{-1} were used for prediction of linearity. It was evaluated by the variance of the regression equation. The coefficient of determination (R^2) was used to explain the total variability of the responses by the linear regression model for the different protease assay models. The LOD and LOQ values were calculated from the calibration curves that defined linearity. Limit of detection, which is expressed in units of concentration, describes the lowest concentration level that can be sort from the blank [46]. The slope of the calibration lines (b) and the standard error of the independent term of the regression (Sb) were used to calculate the LOD and LOQ with reference to IUPAC (International Union of Pure and Applied Chemistry) definition. The

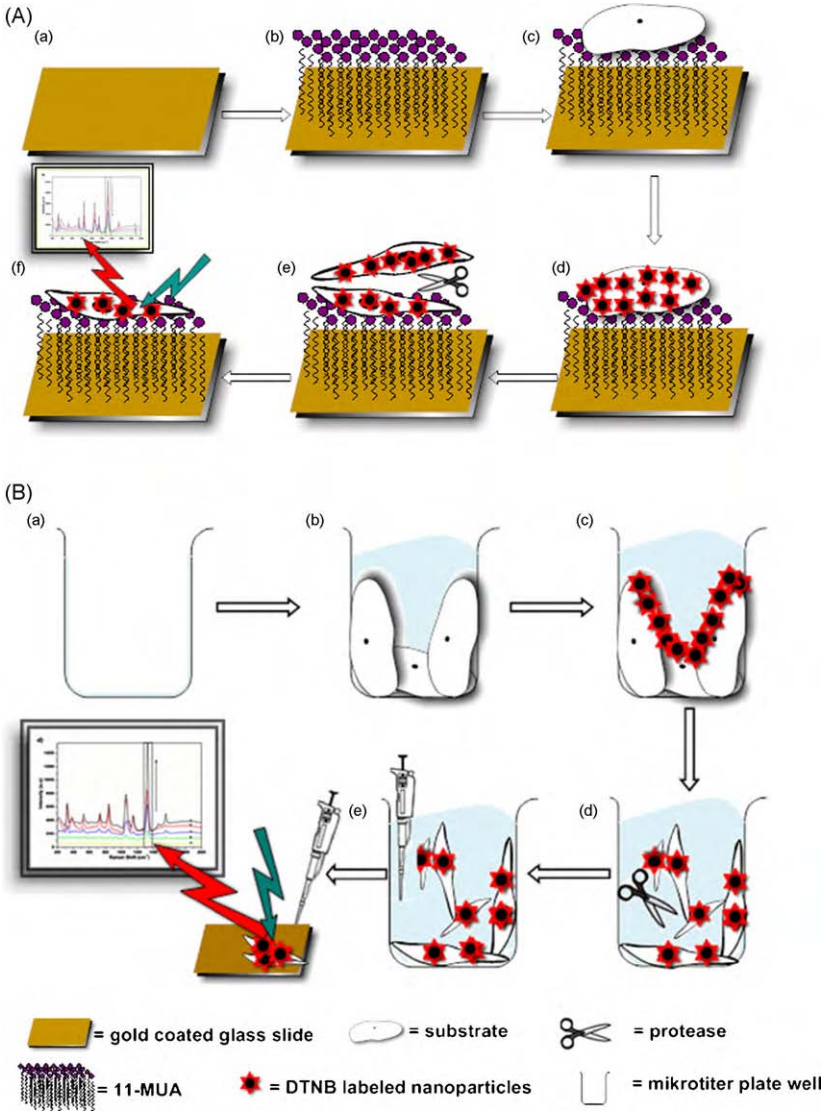

Fig. 1. Schematic illustration of analysis platform preparation; platform I (A), platform II (B). Platform I (a) gold-coated glass slide, (b) constituting SAM on gold-coated glass by 11-MUA, (c) covalent binding of substrate, (d) immobilization Raman labelled nanoparticles on the substrate, (e) enzyme activity and cleavage of the peptide bond and (f) measurement of the remained SERS intensity. Platform II (a) empty microtiter plate, (b) substrate binding by adsorption, (c) immobilization Raman labelled nanoparticles on the substrate, (d) enzyme activity and cleavage of the peptide bond and (e) formation of peptide fragments after enzyme activity and removal of the Raman labelled nanoparticles from the microtiter plate and measurements of the SERS intensity.

equations are:

$$\mathrm{LOD} = \frac{k_1 \times Sb}{b} \quad (1)$$

$$\mathrm{LOQ} = \frac{k_2 \times Sb}{b} \quad (2)$$

k_n is a numerical factor chosen in accordance with the confidence level desired. In this study, k_1 and k_2 values were used as 3.3 and 10, respectively.

The precision (intra- and inter-day repeatability) of the developed method was calculated by making three replicate measurements with the standard solution, containing 135 mU/mL protease, while keeping the operating conditions identical. Raman label intensity was measured in all cases. Repeatability is expressed as the relative standard deviation (RSD%).

Accuracy of the method was tested by quantitative analysis of the commercial enzyme preparate. The results were compared with a colorimetric method using azocasein substrate and RSD value was calculated. At this stage triplicate measurements were performed and averages of the results were used in the calculation.

The developed analysis platform was also used to determine substrate specificity of protease enzyme by using BSA. Instead of the using casein in the preparation stage of platform I, BSA (2%, w/v) was used and the same procedure in the enzyme activity measurement stage was followed. Catalytic powers of protease enzyme in the presence of two different substrates were compared.

2.8. Statistical analysis

The effect of measurements (intra- and inter-day) on band intensity was statistically evaluated by ANOVA using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA). The results are considered to be statistically different at $p < 0.05$.

3. Results and discussion

3.1. Nanoparticle characterization

To investigate the effects of particle shape on analysis performance, two different nanoparticle shapes, spherical and rod, were

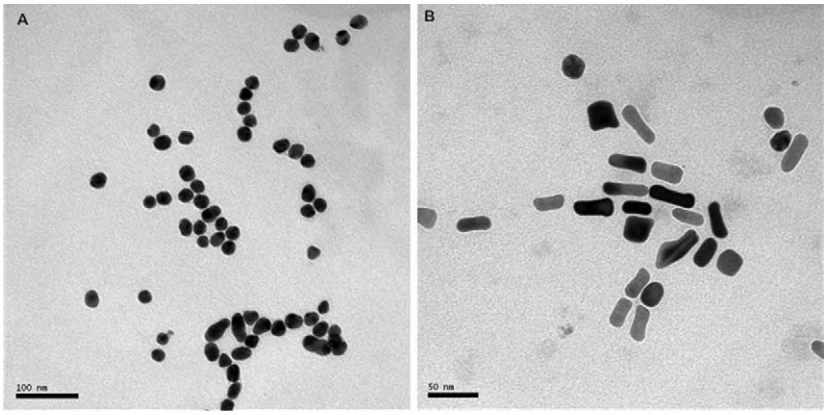

Fig. 2. Transmission electron microscopy (TEM) image of spherical gold nanoparticles (A) and gold nanorods (B).

synthesized in this study. TEM image of spherical gold nanoparticle and gold nanorod are given in Fig. 2A and B, respectively. As shown in Fig. 2A, spherical gold nanoparticles had respective average size of 25 nm. The shape is determined by the aspect ratio of the particles which is defined as the length (~40 nm) of a particle divided by its width (~10 nm). An aspect ratio of synthesized gold nanorods was calculated as approximately 4.

Fig. 3 shows the extinction spectra for each colloidal, which is used to examine the shape of gold nanoparticles. Spherical gold nanoparticles have a plasmon band at 534 nm. On the other hand, the first plasmon band of gold nanorods belongs to transverse plasmon band at 531 nm, and second plasmon band belongs to longitudinal plasmon at 744 nm (Fig. 3). First plasmon is due to the conventional plasmon band for spherical nanoparticles. Second plasmon band appears in the longer wavelength region and it is position depends on the aspect ratio of the rod shaped nanoparticles. This band appears around 600–1600 nm corresponds to the longitudinal plasmon band of rod shaped nanoparticles. As the aspect ratio increases the second plasmon band red-shifted [47]. In our experiment, observed extinction spectra are verified with the TEM images of nanoparticles' size.

3.2. Assay platform preparation

Two assay platforms were prepared by the following procedure given in Fig. 1A and B. Casein coupling on the surface was controlled by fluorescence labeling technique. As seen in Fig. 4, there is no casein coupling on the surface of the platform I before EDC/NHS activation. The surface, which EDC/NHS activation was performed, had high fluorescence intensity of fluorescamine indicates the strong covalent binding of casein on the surface of the platform. And also high fluorescence intensity was observed on the platform II after casein interaction which indicated that casein adsorbed on microtiter surface, successfully.

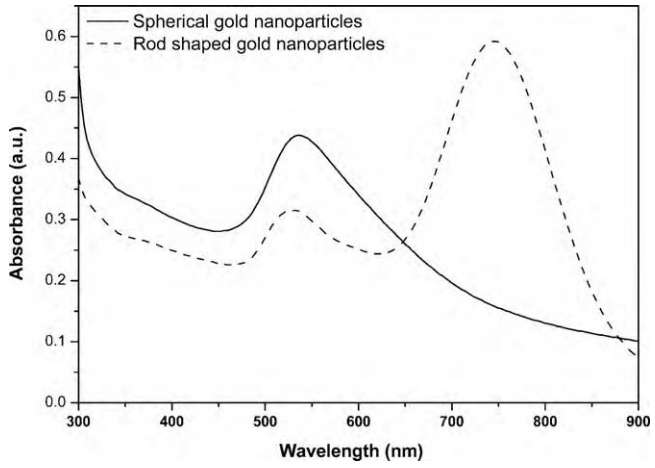

Fig. 3. Extinction spectrum of spherical and rod shaped gold nanoparticles.

3.3. Determination of protease activity based on SERS measurement

In this study, commercially available DTNB was used as the Raman label molecule due to its ability to generate strong Raman signal. Also, DTNB chemisorbs as a thiolate adlayer on the nanoparticle surface and covalently bind to the substrate. Fig. 5 shows the SERS spectra for protease assays conducted by using Raman label constructed from spherical and rod shaped gold nanoparticles. The spectra contain features which are attributable to Raman label and are dominated by bands representative of the DTNB-based adlayer (e.g., the symmetric nitro stretch V_s (NO_2)) at 1326 cm^{-1} and an aromatic ring stretching mode at 1549 cm^{-1} [28]. The Raman spectrum of DTNB's NO_2 stretch is at 1342 cm^{-1} and the aromatic ring stretch is at 1565 cm^{-1} [48]. However, observed NO_2 and aromatic ring stretch is found to be different from the Raman spectrum of DTNB. These changes may arise from the interactions of DTNB with nanoparticles and chemisorbed molecules. Fig. 5A and B represents the decreasing intensity change in platform I depending on enzyme activity. There was a negative correlation between enzyme concentration and intensity. Spectrum of the blanks had the highest intensity. During the hydrolytic reaction catalyzed by protease, the Raman labelled SERS probe which covalently immobilized solid surface via substrate protein became free. The protein and probe were removed by the washing of the solid surface. As expected the remaining intensity of the SERS probe decreased depending on the hydrolytic activity of the enzyme. The intensity values determined by using of different enzyme concentrations with platform II are shown in Fig. 5C and D. Unlike platform I, correlation between enzyme concentration and intensity was positive. In the second platform, a polystyrene microtiter plate was used as support, and the substrate and SERS probe were coated on the well of the plate. As the hydrolytic activity starts, the Raman labelled SERS probe with the peptide fraction was removed from the surface and supernatant solution was used for SERS measurements. For that reason, when the enzyme activity increases on the platform surface, SERS intensity increases due to the increase of free SERS probe in supernatant solution. The lowest intensity of SERS signal was found in blank solution.

Fabricated analysis of the platforms showed good responses with respect to increasing enzyme concentration as illustrated

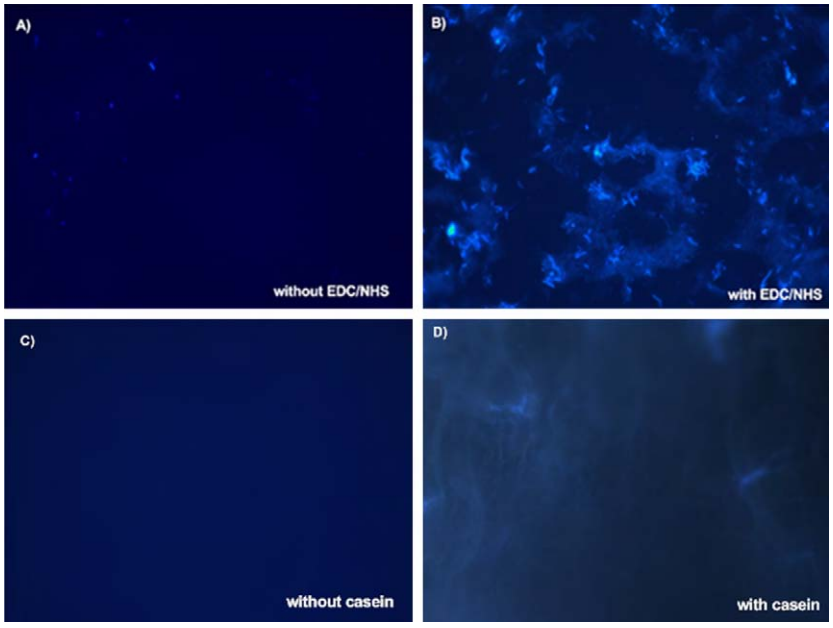

Fig. 4. Fluorescence microscopy analysis of surfaces for platform I: in the absence (A) or in the presence (B) of EDC/NHS and for platform II in the absence (C) or in the presence (D) of casein. Magnified 1000×.

in Fig. 6A and B. Responses obtained from gold nanorod are substantially stronger than those of the spherical gold nanoparticles. When the slopes of the two enzyme concentration–response curves are compared two each other it is seen that the response from the gold nanorod is 2-fold stronger than that of the spherical gold nanoparticles and had a higher aspect ratio (∼4) which is more effective in SERS enhancement.

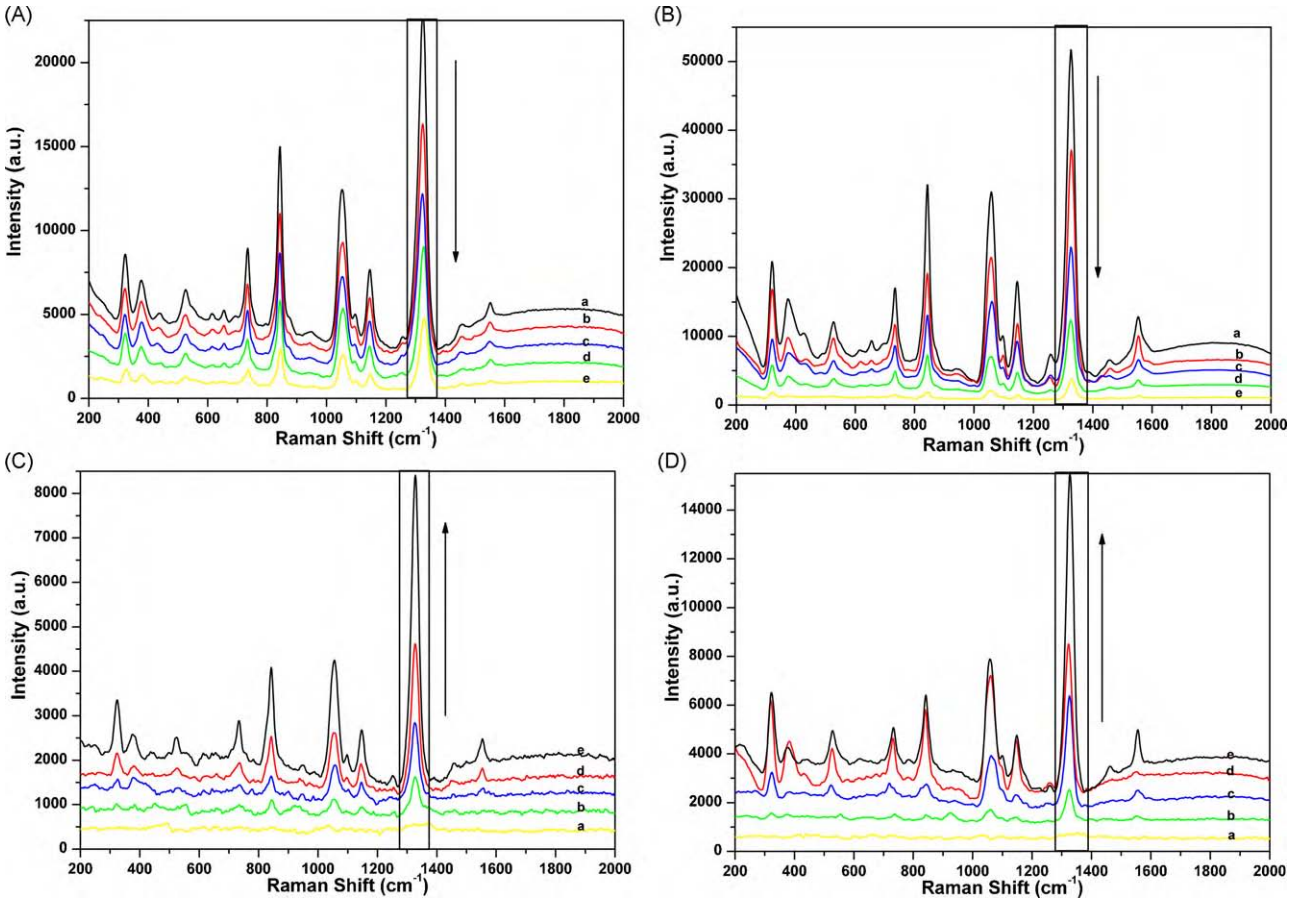

Fig. 5. SERS spectra of protease assays at different enzyme concentrations conducted by using Raman label constructed from spherical (A) and rod (B) shaped gold nanoparticles at platform I or spherical (C) and rod (D) shaped gold nanoparticles at platform II. (a) Blank (phosphate buffer without protease), (b) 67.5 mU/mL, (c) 337.5 mU/mL, (d) 675 mU/mL and (e) 1350 mU/mL protease.

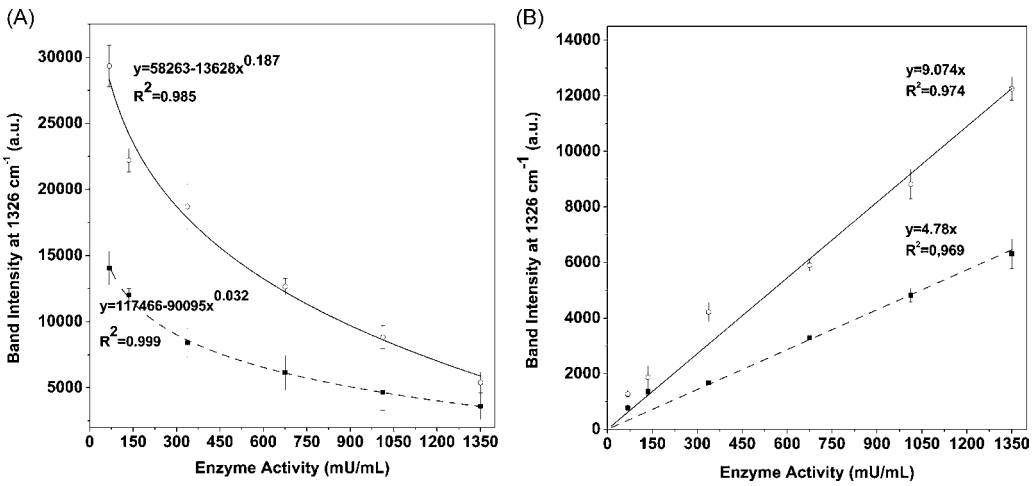

Fig. 6. The enzyme concentration–response curves in the range of 67.5–1350 mU/mL enzyme activity. Platform I (A) or platform II (B) (○) rod shaped gold nanoparticles, (■) sphere shaped gold nanoparticles.

The increase in the SERS enhancement factor with the aspect ratio of silver and gold nanoparticles was also reported in the previous studies which were also confirmed with the results of this study [49–51]. It was reported that cube shaped nanoparticles were found 200 times more sensitive than sphere shaped [52]. However, it was determined that rod shaped nanoparticle was 2-fold sensitive then spherical nanoparticle in the worked aspect ratio. It is indicated that SERS shape has an important effect on the SERS signal intensity. Especially sharp edges of the nanoparticle geometry enhance the SERS intensity. Based on the obtained results, it could be possible to say that assay platform I with rod shaped SERS probe had better results than the other combinations. For this reason, validation of the study was performed using the platform I with rod shaped SERS probe.

Fig. 7 shows a plot of SERS intensity as a function of enzyme concentration for platform I with rod shaped SERS probe. The relationship between SERS signal intensity and enzyme concentration was described with a power function. A nonlinear least-squares fit to the data using a power law formula is shown in Fig. 7, indicating that the SERS intensity was equal to the enzyme concentration to the power of 0.201 and a coefficient of 12,071 (after subtraction of the blank SERS intensity). The correlation coefficient, R^2, was equal to 0.987 within the range of 0–1350 mU/mL enzyme concentration. As expected, the change in the SERS intensity decreased with increasing of the enzyme concentration on the platform I. Observed change was linear up to 2 mU/mL enzyme concentration ($y = 46785 - 1521x$ $R^2 = 0.979$). Over this concentration, a deviation from the linearity was observed and the change in the SERS signal intensity gradually decreased with increasing enzyme activity due to the insufficient SERS label in the platform. In other words, the enzymatic reaction rate is controlled by the enzyme activity in a low enzyme concentration; however, it is determined by SERS label in the high enzyme concentrations. As seen in the calibration curve of the activity measurement (Fig. 7) the more accurate results were obtained with a low concentration of the protease enzyme. The inset of Fig. 7 shows the linear range of the analysis platform. At the beginning of the hydrolysis, when the amount of SERS label is in substantial excess to the amount of enzyme, the reaction rate shows

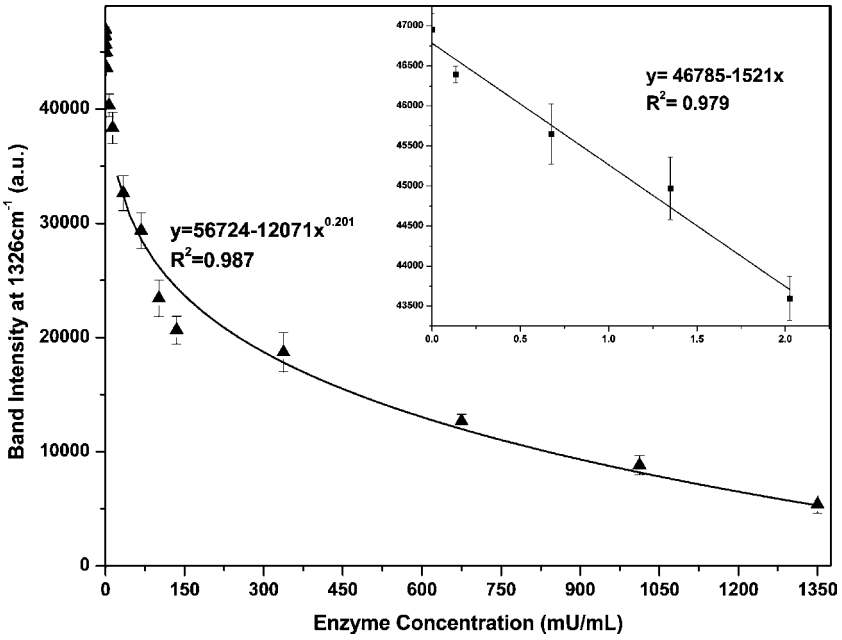

Fig. 7. The correlation between the protease concentration and SERS intensity with rod shaped SERS probe using platform I.

Table 1
Intra-day and inter-day repeatability of the proposed procedure (Enzyme concentration is 135 mU/mL).

Intra-day		Inter-day	
Measurements	Band intensity (a.u.)	Days	Band intensity (a.u.)
1	20,178	1	20,900
2	19,401	2	19,589
3	20,630	3	20,856
		4	22,106
		5	20,811
		6	21,751
		7	21,386
Average	20,070		21,057
SD[a]	507.5		753.2
RSD[b]	2.53		3.58

[a] Standard deviation.
[b] Relative standard deviation (RSD (%) = (SD/mean) × 100).

first order kinetic. When the hydrolysis continues the reaction occur at zero order kinetic because of the insufficient SERS label.

The proposed method is a sensitive method to determine activity of commercial enzyme. The method can be applied for the determination of enzyme activity with minimal sample preparation stage and in a short analysis (less than 40 min) time which includes incubation period of enzymatic reaction (30 min), SERS measurement, and other activities (less than 10 min). The use of an assay platform designed in this study is applicable to measure the true activity of protease enzyme with its substrate.

3.4. Validation

Based on the optimal design of the protease assay, the method presented higher linearity in the enzyme activity range from 0.1 to 2 mU/mL. Each point in the final calibration curve corresponded to the mean value of three independent measurements. LOD and LOQ values of the developed protease assay were calculated as 0.43 and 1.30 mU/mL, respectively. When the LOD value of the developed method was compared with the method in the literature it is possible to say that the developed method was one of the most sensitive method for the detection of the protease activity [53,54].

Repeatability of the proposed procedure was investigated and the results were given in Table 1. The results of intra-day repeatability expressed as RSD% value were calculated as 2.5% for 135 mU/mL of protease concentration. Furthermore, the analysis of protease standard solutions on seven different days showed adequate values of precision (RSD value was calculated as 3.6% for inter-day repeatability) considering SERS intensity values. The effects of intra- and inter-day measurements on band intensity values were found to be statistically insignificant ($p > 0.05$).

Accuracy of the method was tested by quantitative analysis of the commercial enzyme preparate that is used in the cheese making process. The enzyme activity was calculated using the calibration curve and was found as 29.4 mU/mL. The result was in agreement with the azocasein method (27.8 mU/mL). The RSD% value of SERS measurement for commercial enzyme preparate was found to be 1.1%. Since the results were within the acceptable range of ±5.0%, the method is deemed to be accurate.

The usability of the method for investigation of substrate specificity of the protease was also tested. BSA was used as substrate instead of casein in the preparation of the analysis platform and the same analysis procedure was followed to determine the protease activity. A calibration curve was also prepared for the assay platform in which BSA was used as substrate. The slope of the calibration curve was compared with the assay platform which casein was used. The results indicated that the protease preferred casein rather than BSA and specific activity of the protease toward the casein was 2.6-fold higher than that of the BSA (data not showed).

4. Conclusion

In this study, a simple, rapid, and sensitive method for the quantification of protease activity based on SERS measurements is reported. The method was successfully applied to determine enzyme activity in commercial enzyme. The requirement of minimal sample preparation stage was the important issue in this study and only a small volume of sample was enough to determine protease activity with high sensitivity and selectivity due to SERS. This method can be used for a wide range of protease enzyme activity determination with minimal sample preparation stage and in a short analysis time. The presence of several common chemicals encountered in biological buffers and foods can be tolerated in the assay without interference effect of the compound on the response. Unlike in some other methods, extraction procedure is not needed to eliminate the interference of the compound present in the sample medium. Therefore extraction procedure is not needed. Enzymatic reaction is also performed in a specific analysis medium. The catalytic power of the enzyme is very sensitive to the reaction medium. For that reason its actual activity is the activity determined in the real reaction medium. Enzyme activity determined in a different analysis medium is not equal to the true activity of the enzyme. Proteases occur naturally in all organisms and it is involved in digesting long protein chains into short fragments. On the other hand, its affinity towards the substrate varies depending on the type of the substrate. Based on this knowledge its catalytic activity should be determined with the substrate which is used in the real reaction medium. However, it is not possible to do that for most of the substrates. In practice, some common substrates such as casein and gelatine are used for determination. The assay platform designed in this study is applicable to measure the true activity of protease enzyme with its substrate. Protease activities with two different substrates (casein and BSA) were monitored in the present study. The proposed method has a flexibility to try different substrates for the detection of various enzyme activities. Amines, carboxylic acids and thiols in corresponding amino acid residues are most commonly used for substrate immobilization. Also, avidin–biotin interaction can be used to immobilize the nanoparticles to the surface platform via substrate. Thus, with slight modifications, this procedure can be applicable for different enzymes.

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