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Protein & Peptide Letters, 2021, *28*, 809-816

RESEARCH ARTICLE

Assessment of Proteolysis by Pyrylium and Other Fluorogenic Reagents

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ARTICLE HISTORY

Received: July 27, 2020
Revised: November 04, 2020
Accepted: November 09, 2020

DOI:
10.2174/0929866528999201231214954

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Abstract: ***Aims*:** We aim to evaluate the potential application of amine reactive fluorogenic reagents for estimating enzymatic proteolysis.

***Background*:** Proteolytic enzymes play important roles in regulating many physiological processes in living organisms.

***Objectives*:** Assessment of protein degradation by using reagents for protein assay techniques.

***Methods*:** We have assayed samples at the start and after 30-60 minutes incubation with trypsin by Chromeo P503 (Py 1 pyrylium compound) and CBQCA (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) as amine reactive reagents and NanoOrange as non-amine reactive dye.

***Results*:** All BSA prepared samples with trypsin have shown significantly higher fluorescence intensity (FI) *versus* controls (which reflects proteolysis) when assayed by Chromeo P503 (Py 1 pyrylium compound) and CBQCA (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) as amine reactive reagents. However, same samples assayed with NanoOrange as non-amine reactive reagent did not show any significant variation between samples containing trypsin and controls.

***Conclusion*:** These results are confirming reliability of highly sensitive protein assays utilizing amine reactive fluorogenic reagents for general estimation of proteolysis.

Keywords: Proteolysis, spectrofluorometry, pyrylium, CBQCA, chromeo P503, nanoOrange.

1. INTRODUCTION

Proteolysis is a hydrolytic reaction of peptide bonds in which proteins are cleaved into smaller peptides or into amino acid residues. Most proteolytic reactions are catalyzed by either chemicals or enzymes. Proteolytic enzymes naturally exist intracellularly and extracellularly posing a well-regulated cleaving system. In addition, proteolytic enzymes are implicated in modulating the activity of bioactive proteins [1-5]. Proteolytic enzymes play important roles in regulating physiological and cellular processes of living organisms such as food digestion, autophagy which prevented the accumulation of unwanted or abnormal proteins within the cells, activation of proteins to attain their function and in human diseases such as autoimmune diseases, bacterial infections, cancer, and viral diseases [6, 7]. Their implication in the various biological processes has attracted more attention for their therapeutic potentials and applications in medical, pharmaceutical and biotechnology research [8-10]. For example, recombinant therapeutic protein may undergo limited proteolysis during cell culture processes and the contamination of proteases may modify or degrade recombinant proteins during formulation affecting their stability [11].

The major challenges in this research area are to identify these enzymes, their specific substrates and proteolytic products, and to define their specific activity to better understand their physiological functions and to predict their role in pathological conditions. Several classes of proteolytic enzymes in living systems were characterized according to their cleavage location (endo- and exo-peptidases), specific site cleavage (*e.g.* serine, glutamic, aspartic, threonine, cysteine proteases), and optimal pH for their activity (alkaline or acidic proteases) [12, 13]. In addition, some proteolytic enzymes such as (cyclins, renin, cathepsin, *etc.*) have specific cleaving activity and modulate functional bioactive proteins that are implicated in specific regulatory mechanisms [1-5]. We used trypsin in our study which is a serine endopeptidase enzyme that cleaves peptide bonds in which serine amino acid acts as a nucleophilic residue at the enzymes active site with an optimal activity at 8.5-8.7 pH and 40°C temperature of media [14, 15]. Activity of some proteolytic enzymes also depends on the presence of metal ions and ionic strength of reaction media [5].

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Protein & Peptide Letters

Nevertheless, researchers are applying protocols and techniques by performing operations far away from the optimal conditions for the activity of proteolytic enzymes to avoid proteolysis or providing protection of protein samples by adding stabilizers or protease inhibitors [16].

Various bioanalytical techniques have been employed to assess proteolysis and estimate activity of enzymes in samples. Most analytical approaches for proteolysis evaluation are based on the estimation of N-termini in samples. Early attempts have applied spectrophotometric techniques by using amine reactive compounds for estimating newly generated amine termini (neo-N-Termini) as proteolysis indicators [17-19]. Erlanger, Zimmerman and their colleagues have also generated chromogenic and fluorogenic substrates by combining chromophores or fluorophores with specific amino acid sequences to assess catalytic activity of specific digestive enzymes [20-22]. Later studies have been based on more sensitive techniques by using isotope labeling for identification of neo-N-termini and estimation of protease activity [23, 24]. Protein substrates like azoalbumin, azocasein, azocoll, azogelatin, green fluorescent protein (GFP) and fluorescent hemoglobin with chromogenic property or fluorescence resonance energy transfer (FRET) capability upon protein hydrolysis are found at life science companies and used for general estimation of protease activity in biological samples [25-27]. However, using these products in assays may require separation of proteolytic fragments from protein substrates [25].

Proteolysis assessment in biological samples has been demonstrated previously, and some studies have indicated their correlation with specific pathological conditions. Increased proteolysis in stool samples has been correlated with inflammatory bowel disease and microflora colonizing the colon [28-31]. Estimation of proteolysis and the significance in urine have been demonstrated in diabetic patients with nephritis [32, 33]. Upregulation of proteases in serum and other body fluids have been demonstrated in hemorrhagic disorders [34]. Well correlations of serum protease and caspase activity were observed in advanced stages of breast malignancy [35]. Various studies were also conducted on serum for identification of circulating proteolytic products as biomarkers for clinical application in early diagnosis or therapeutic evaluation in cancers [35-39].

Proteomic research on proteolytic enzymes in the living systems requires more sensitive and sophisticated tools for the identification of their specific substrates, proteolytic products and characterization of enzymes' activity [38, 40-44]. These advanced techniques combined with the use of mixture of substrate sequence libraries are suggested for studying kinetics and defining proteolytic specificity of enzymes and their interactions [44]. Recently, researchers are calling for multiplexed characterization of proteolytic biomarkers combined with genetic biomarkers to improve sensitivity and specificity of the diagnostic tools, and to optimize management of cancer diseases [39, 45].

Preliminary assessment of enzymes' activity would require utilization of rapid and simple methods that are sensitive and reliable. We report a highly sensitive method using amine reactive fluorogenic reagents for general estimation of proteolysis which could be helpful for preliminary estimation of proteolytic activity and indicative tool for hydrolysis in stored protein samples or for general estimation of enzymes' activity in biological products.

2. EXPERIMENTAL SECTION

2.1. Preparation and Collection of Samples

Two methods for incubation of samples with trypsin having differences in reaction buffers, incubation period and temperature were employed. BSA (Capricorn Scientific GmbH, fraction V, origin USA, Ebsdorfergrund, Germany) was prepared as 4mg/ml stock in distilled water and used as substrate for the activity of trypsin enzyme in both methods.

2.1.1. Preparation Method 1

Different amounts (0, 25, 50, 100 and 200 µl) of trypsin EDTA 1x (0.05%) in PBS, w/o Ca^{2+}, w/o Mg^{2+} (EuroClone S.P.A., Italy) were placed in five Eppendorf tubes, and volume accomplished to 300 µl by Dullbecco's Phosphate buffered saline (PBS), w/o Ca^{2+}, w/o Mg^{2+} (EuroClone S.P.A., Italy, pH: 7.4) to obtain samples with four relative concentrations of trypsin (1x, 2x, 4x and 8x) and a control (0) without trypsin. After vortex, 200 µl BSA stock was added to all tubes and vortexed. The final concentrations of trypsin in reaction tubes were 0.000, 0.0025, 0.005, 0.01 and 0.02% for controls and the aforementioned relative concentrations, respectively. Then 200 µl from each tube were collected and fixed with 200 µl of dimethyl sulfoxide (DMSO) (AppliChem, GmbH, Darmstadt, Germany) and stored at -20 until the assay. This set was considered as collected samples within 2-3 minutes of preparation and before incubation. The remaining amounts of prepared samples were incubated for 60 minutes at 37°C. After vortex, 200 µl were collected again from each tube, fixed and stored as described above until assay. These samples were considered as collected samples after incubation at 37°C. Additional quadruplet set of samples with 200 µl trypsin and BSA were prepared in PBS as described above in Eppendorf tubes. The content from each tube from the second set was divided into 2 tubes containing 200 µl each for preparing two subsets of samples. One subset was incubated at room temperature and the other at 37°C for 60 minutes. After incubation, samples were fixed and stored as described above until assay. All collected samples using this method were analyzed for protein content with Chromeo P503 (Py1) (Santa Cruz Biotechnology Inc., Dallas, USA).

2.1.2. Preparation Method 2

Reactions for this set of samples were carried out in Bicarbonate Carbonate Buffer (BCB) (AppliChem, GmbH, Darmstadt, Germany) at room temperature with reduced incubation time to 30 minutes. Changes in protocol were made to ensure working at the submaximal activity of enzyme. To prepare samples, in each experiment, 25, 50 and 100 µl trypsin were placed in three different Eppendorf tubes, following by addition of PBS to total volume of 100

µl. Two additional Eppendorf tubes were prepared; one with 100 µl PBS without trypsin and the other with 100 µl trypsin to serve as controls. Then, BCB (0.2M, pH=8.5) was added to total volume of 400 µl and vortexed. 100 µl of BSA stock was added to tubes containing 0, 25, 50 and 100 µl trypsin then vortexed. Distilled water was added to the tube containing 100 µl trypsin without BSA to total volume of 500 µl and vortexed. Immediately after mixing, 200 µl were collected from tubes containing BSA, fixed with 200 µl of diluted DMSO in distilled water (1:1 v/v) and stored at -20 until the assay. Another collection of samples was made after 30 minutes of incubation at room temperature using the same volume as above from all prepared tubes, then fixed and stored. Collected samples before and after incubation were analyzed by Chromeo P503, CBQCA (Invitrogen, Eugene, USA) and NanoOrange kit (Invitrogen, Eugene, USA).

2.2. Protein Assay

Collected samples were analyzed using pyrylium dye (Chromeo P503), CBQCA kit and NanoOrange, highly sensitive fluorescent techniques for determination of protein content. Methods with pyrylium and CBQCA are based on detection of reactive N-terminal amines [46-48], while the latter technique is based on coating protein with a detergent and estimation of protein-detergent complex in samples [49].

As fluorogenic amine reactive pyrylium 1 (Chromeo P503^{Tm}) was mainly used to estimate changes in N-termini by measuring fluorescence intensity (FI) in samples after reaction with dye. Briefly, dye stock was prepared by dissolving 1mg of dye in 2ml DMSO. Then, reaction with dye was performed in small volume by adding 5 microliters to Eppendorf tubes containing 10 µl of sample solution from the first set or 20 µl from the second in 50 µl bicarbonate carbonate buffer (BCB) (0.1M, pH≈9.2). After one hour reaction at room temperature in dark and shaking at 45RPM, the volume was reconstituted to 1ml with distilled water.

Additional assays on the second set of samples by CBQCA and NanoOrange techniques were employed to confirm the obtained results. Since we have observed the need for changing protocol of samples' preparation to avoid reaching the maximal proteolytic effect obtained after one hour incubation with enzyme in the first protocol, we have not repeated the assay in the first set by CBQCA and NanoOrange kits. The amine reactive reagent from CBQCA kit was dissolved in 410 µl DMSO (40 mM) as recommended by manufacturer and aliquoted as stock. Just before use, the dye was diluted to 2 mM concentration in borax buffer (0.1M, pH = 9.3). Samples were assayed according to manufacturer protocol. NanoOrange kit was used in the assay as recommended by manufacturer. Both CBQCA and NanoOrange protein quantification assays were completed with 10 µl of collected samples.

Along with CBQCA and Chromeo P503 assays, in addition to controls containing reagent only, two additional tubes were prepared from control samples before incubation. One tube contains half volume of prepared sample and the other tube has double volume to generate reference concentration curves for these reagents with protein in our samples. For the same purposes, eight tubes were prepared from two different experiments and assayed with NanoOrange (four tubes were with half volume and other four tubes with double volume of samples). In addition, to eliminate any potential variations in fluorescence intensity (FI) in assays due to presence of trypsin enzyme, controls with the highest concentration of trypsin without protein samples were prepared.

2.3. Spectrofluorometry

Following 1h reaction of samples from the second set with dyes, fluorescence intensities (FIs) were measured by Shimadzu RF-5301PC spectroflourometer (Kyōto, Japan), equipped with spectroscopy software RFPC-version 2.04. Slit widths for excitation and emission were set at 5 nm, and wavelengths according to fluorescence properties of each dye. About 400 µl of prepared samples were used for quantitative measurements of fluorescence intensities in rectangular quartz cuvettes (2x10mm) with metallic adaptor at λexc/em = 503/600 nm for Chromeo P503, 465/550nm for CBQCA and 485/595nm for NanoOrange samples. Fluorescence intensity was also read in 96 well plates for the first set samples with Chromeo P503 by Synergy HTX Biotek plate reader (Vermont, USA), equipped with filter sets (λexc = 485/20nm and λem = 590/35nm) and software version: 2.07.17.

2.4. Data Analysis

All fluorescence Intensity readings from both devices were exported to Microsoft Excel worksheet 2010 for calculation of means, standard deviations, standard error of measurement (SEM) and *p* values. Statistical significance was considered when *p* value is <0.05.

3. RESULTS

In the first set of samples, fluorescence intensities (FI) were measured using plate reader for samples taken in the first 2-3 minutes after addition of different concentration of trypsin and after one hour incubation at 37°C are shown in Figure **1**. In these measurements, as trypsin concentration increased, higher FI was recorded. Means for FI±SEM (X1000 RFU) were 54.7±2.4, 70±4.2, 77.4±5.7, 90.5±3.7 and 93.9±1.8 with 0, 25, 50, 100 and 200µl of trypsin respectively and *p* <0.005 in samples with the lowest concentration of trypsin *vs* controls (Figure **1A**, n=5). All samples incubated with trypsin at 37°C have also shown significant increase in FI. Means in FI±SEM were 80.7±4.7, 82.5±2.4, 81.7±2, 74.3±4.3 for the above concentrations respectively *vs* 58.1±3 in incubated control (*p* <0.005 – <0.02, Figure **1A**, n=5). Even with the lowest values of FI at the highest concentration of trypsin, we still observed statistical significance in the collected data for samples incubated with trypsin *vs* controls (*p* <0.01, n=5). Similar results were observed in samples analyzed in quartz cuvettes by Shimadzu RF-5301PC spectrofluorometer (Figure **1B**).

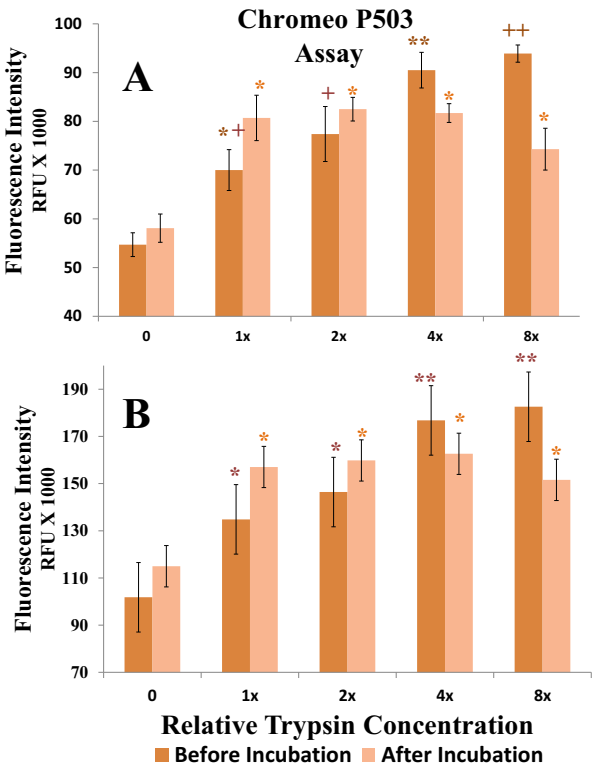

Figure 1. Assay of BSA with Chromeo P503 after one hour digestion by trypsin at 37°C. Mean records for FI (RFU X1000) read by **A**: Synergy HTX Biotek plate reader and **B**: Shimadzu PC-5301 for samples assayed with Chromeo 503 collected (before incubation) within 2 minutes after addition of trypsin (darker columns) and after one hour incubation at 37°C (lighter columns). The X axis showing relative volumes of trypsin added. The first pair of columns is control samples with no Trypsin added. Pairs of columns (2-5) show FI in samples treated with 25, 50, 100 and 200 µl trypsin (1x, 2x, 4x and 8x respectively) (n=5). (*p* values were (in **A** <0.02 and <0.003 for 8x *vs* 2x and 1x respectively and <0.01 for 4x *vs* 1x and in **B** <0.02 and <0.001 for 8x *vs* 2x and 1x respectively and <0.04 and <0.02 for 4x *vs* 2x and 1x respectively). ***, +, **** and ++ for statistical significance.

New set of samples were prepared in BCB and incubated at room temperature for 30 minutes. In addition to control tubes without trypsin, three tubes with 25, 50 and 100 µl of trypsin were prepared. Protein assays with Chromeo P503, CBQCA (as amine reactive dyes), and NanoOrange kit (for general protein assay) were performed. FI were recorded by Shimadzu RF-5301PC spectroflourometer for all reagents. Results of this study are shown in Figure **2**. Use of 20 µl of sample with Chromeo P503 probe resulted in significantly higher FI in all samples containing trypsin regardless of the reaction time. Samples were collected within 2 minutes and after incubation for 30 minutes at room temperature (Figure **2A**). FI for samples collected within 2 minutes and after adding 25, 50, and 100 µl trypsin were 108.8±4.8, 120.6±4.3, 133±3.9 (X1000) RFU respectively *versus* 84.5±1.7 (X1000) RFU in control tubes without trypsin (*p* values were <0.002 - <0.03, n=4). After incubation for 30 minutes FI were 109.8±4.2, 122.2±6.6, 125.3±2.9 (X1000) RFU in samples containing trypsin *versus* 82±2.7 (X1000) RFU in controls without trypsin (*p* <0.001 - < 0.02, n=4). Statistically significant difference was observed between samples containing 100 µl trypsin and those treated with 25 µl in both groups (*p* <0.02). However, no significant differences were obtained for replicates collected within 2 minutes and incubated samples for 30 minutes at room temperature.

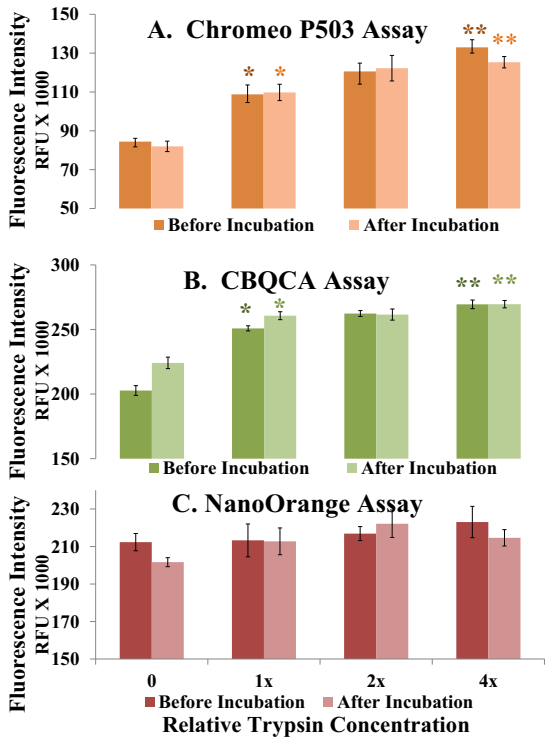

Figure 2. Assay of BSA with Chromeo P503, CBQCA and NanoOrange after 30 minutes digestion by trypsin at room temperature. Fluorescence Intensity (FI) (X1000 RFU) in collected samples (before incubation) within 2 minutes after addition of trypsin (darker columns) and after 30 minutes incubation at room temperature (lighter columns). The X axis is showing relative volumes of trypsin added. Samples were assayed with Chromeo P503 (**A**), CBQCA (**B**) and NanoOrange (**C**) reagents. The first pair of columns is control samples. Columns' pairs (2-4) are FI in samples treated with 25, 50 and 100 µl (1x, 2x and 4x) trypsin, respectively (n=4). Significances in 2A and 2B (* and **) were considered when *p* value < 0.05 (1x *vs* controls (*) and 4x *vs* 1x (**). FI records were read by Shimadzu RF-5301PC Spectrofluorometer.

Samples analyzed using CBQCA have also shown significant increase in FI after addition of trypsin in comparison to controls (Figure **2B**). In samples collected within 2 minutes after adding 25, 50 and 100 µl trypsin, FI were 250.9±2, 262.4±2.3, 269.5±3.3 (X1000) RFU respectively *vs* 202.8±3.8 for controls (*p* <0.001, n=4). Significant differences were also observed for samples containing 4x and 2x trypsin concentrations compared to

samples containing 1x trypsin (p <0.01 and <0.04 respectively). Samples incubated for 30 minutes also showed higher FI with significant statistical differences compared to controls. FI for samples containing trypsin were 260.7±3.1, 261.6±4.3, 269.6±2.9 (X1000) RFU, whereas FI for controls were 224.2±4.4 (X1000) RFU (p <0.01 - <0.02). However, among samples containing trypsin statistical significance was obtained only between samples containing 4x and 1x trypsin (p <0.02). Although no significant differences were observed among replicate measurements with samples containing trypsin (P >0.05), we have observed significantly higher FI in controls after 30 minutes of incubation at room temperature (224.2±4.4 *vs* 202.8±3.8 (X1000) RFU, p <0.003, n=4).

Samples analyzed with NanoOrange, a non-amine reactive dye, no significant statistical differences in FI were obtained between trypsin treated samples and controls. After adding trypsin, samples collected within 2 minutes and after incubation for 30 minutes at room temperature showed almost identical FI (Figure **2C**). FI records were (212.3±4.6, 213.3±8.8, 216.9±3.8, 223.1±8.4) in samples collected within the first 2 minutes of reaction and (201.7±2.4, 212.8±7.2, 222.1±7.3, 214.6±4.4) in collected samples after 30 minutes incubation with 0, 25, 50 and 100µl trypsin respectively (p >0.1, n=4). All these results were not corrected for the background fluorescence signals caused by the presence of the reagent alone in the reaction buffer.

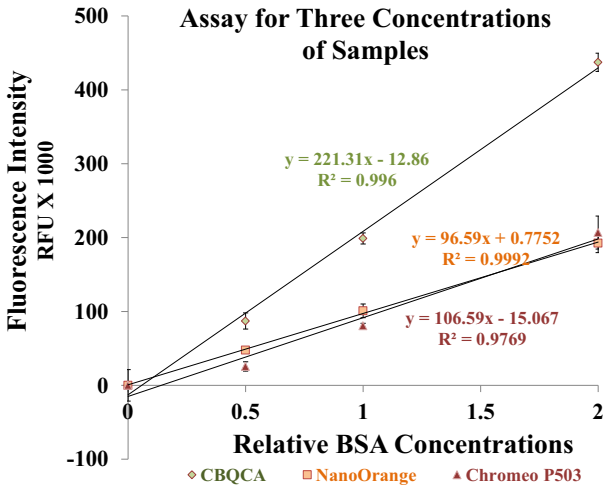

Figure 3. Assay of three concentrations from pre-incubated BSA controls by Chromeo P503, CBQCA and NanoOrange. Fluorescence Intensities for three concentrations from control samples without trypsin collected within 2 minutes of incubation (half, controls, double protein amounts) with reagents used for protein assay. For assays by Chromeo P503: 2x volumes of BSA were used than by other reagents. In these assays, FI in assay buffer for reagents were subtracted from FIs values of each respective record. R^2 for the curves were 0.996, 0.999 and 0.977 for CBQCA, NanoOrange and Chromeo P503 respectively, (n=4 for each reagent). Bars reflect STDEV. FI records were read by Shimadzu RF-5301PC Spectrofluorometer.

Two additional concentrations (half and double) from samples collected within 2-3 minutes were assayed as a quality control for the reagents used in this study. In these experiments FI were corrected based on the background signals of reagents in assay buffers. Results of these assays are showing concentration dependent increase in FI with all reagents used in our study with R^2 of >0.97 (Figure **3**), indicating the high accuracy of FI signal readings. However, the slope of the line was higher for CBQCA than NanoOrange or Chromeo P503 assays, which may indicate the differences in sensitivity between reagents in assays. The background FI signals for reactive dyes were high for NanoOrange and very low for Chromeo P503 and CBQCA (111X1000 RFUs *vs* 4-5X1000). In addition, we have also assayed the highest concentration of enzyme using sample preparation method 2. In these assays, minimal variations were observed in FI *versus* background signals for reagents in reaction buffers ((0.15, 3 and 7) X1000 RFU for Chromeo P503, CBQCA and NanoOrange, respectively).

4. DISCUSSION

Methods based on fluorogenic amine reactive dyes used for protein assays represent a reliable approach for analyzing proteolysis products in samples. Upon proteolytic degradation of protein, the number of molecules in sample having free N-terminal amino groups is increased, which, in turn, corresponds to the number of proteolytic products [17-19]. Evaluation of digestive enzymes activity in biological samples were traditionally performed using fluorogenic or chromogenic substrates which generate labeled peptide fragments with fluorophores or chromophores that can be detected in the supernatant of reaction media [25].

In our study, detection of peptide fragments, resulting from proteolytic degradation of protein, was achieved by using highly sensitive fluorogenic dyes capable of reacting with primary amines of the obtained fragments. We have detected protein fragments using Chromeo P503 and CBQCA assay kits, highly sensitive amine reactive fluorogenic reagents, in samples containing the same amounts of BSA and incubated with different concentrations of trypsin. Samples analyzed by Chromeo P503 kit and using Biotek plate reader have shown significant increase in FI in all trypsin treated *versus* controls collected before and after incubation for 1 h at 37°C in PBS (Figure **1**). No significant differences in FI were observed among samples incubated at 37°C and containing 25, 50, 100 and 200 µl (1x, 2x, 4x and 8x) of trypsin, respectively. However, concentration dependent increase was observed in samples collected within first minutes of incubation and showing higher FI. Statistically significant FI values were obtained for samples containing 200 µl trypsin compared to those with 50 µl and 25 µl of trypsin and for samples containing 100 µl trypsin compared to those with 25 µl of trypsin (p values were <0.02, <0.003 and <0.01 respectively, Figure **1A**, n=5). Similar results were observed when Shimadzu RF-5301PC spectrofluorometer was used (Figure **1B**).

Since we have observed a significant decrease in FI in samples incubated with the highest concentration of trypsin compared to collected samples within 2-3 minutes of incubation (p <0.003), additional experiments were performed in quadruplets with the highest concentration of trypsin tested and divided into two groups. One group of samples was incubated for 1 h at room temperature and the second group was incubated for 1 h at 37°C. After performing the assay in duplicates using Chromeo P503 kit, we have observed a significantly lower FI in incubated tubes at 37°C for one hour *versus* tubes incubated at room temperature (72.6±3.5 *vs* 86±1.7, p <0.04). However, no statistical differences were observed in FI among samples that belong to these two groups recorded by Shimadzu RF-5301PC Spectrofluorometer.

Concentration dependent increase in FI in collected samples within first minutes of incubation was observed, reaching reaction rate plateau with all concentrations of trypsin using sample preparation method 1. Thus, we performed reaction at room temperature and reduced incubation time to 30 minutes. These changes in protocol were made to ensure proteolytic reactions at the submaximal activity of enzyme. In addition, we have used bicarbonate carbonate buffer (BCB: 0.2M, pH: 8.5) instead of phosphate buffer saline (PBS) with optimal pH condition for the activity at three different concentrations of trypsin (25 (1x), 50 (2x) and 100 (4x)). The set of samples collected from the second protocol was prepared for analyzing protein concentrations using Chromeo P503, CBQCA kit (as amine reactive reagents) and NanoOrange (which estimates coating detergent adsorbed to protein), and Shimadzu RF-5301PC Spectrofluorometer with wide range spectral recording.

Similar results were also obtained in this set of samples using Chromeo P503 kit. This assay showed significant differences in all samples containing trypsin compared to controls (p <0.03 with the lowest concentration of trypsin in samples collected within first minutes of incubation and p <0.003 in incubated samples for 30 min., Figure **2A**, n=4). Significant differences were observed among samples containing the highest (4x) and the lowest (1x) trypsin concentrations before and after incubation for 30 minutes (p <0.02, n=4). Samples analyzed by CBQCA kit showed higher FI values than those analyzed by Chromeo-P503 kit, indicating differences in the sensitivity of assays performed using these two reagents. Significant increase in FI was observed in samples with the lowest concentration of trypsin compared to controls (p <0.0002 and <0.02 in samples collected within first minutes and after 30 minutes incubation respectively, Figure **2B**, n=4). Also, significant differences in FI were observed between the highest and the lowest concentrations of trypsin (p <0.006 for FI values in samples collected within 2 minutes of incubation and p <0.02 for values after 30 minutes incubation at room temperature). In contrast, no significant differences were observed between the same pairs of trypsin concentrations (before and after incubation) in all assays carried out by Chromeo P503 and CBQCA kits. This observation may indicate that the maximal effect of trypsin achieved within the first few minutes of incubation. Control samples analyzed by CBQCA kit showed approximately 10% higher FI values after incubation for 30 minutes *versus* before incubation (Figure **2B**, p <0.003, n=4). However, this variation was not observed in samples analyzed by Chromeo P503 kit in PBS or in BCB media.

Although we have much higher background fluorescent signal in buffer without protein using NanoOrange kit compared to Chromeo P503 and CBQCA kits, insignificant variations in FI were observed for NanoOrange kit in samples containing trypsin and collected within 2-3 minutes and after 30 minutes of incubation (Figure **2C**, n=4). In addition, we have excluded the potential interferences that could result from adding trypsin to solutions by assaying control samples containing highest concentration of trypsin. Compared to FI for samples collected within first minutes of incubation, the variations in FI in samples containing highest concentration of trypsin without BSA were minimal in all assays and could account for less than 2% of FI increase using CBQCA and Chromeo P503 kits and for approximately 4% of FI increase using NanoOrange kit. Nevertheless, the increase in FI in samples containing BSA and the highest concentration of trypsin was approximately 30% in assays with CBQCA kit and more than 50% when Chromeo P503 kit was used.

Instead of generating concentration curves to eliminate experimental errors in our assays, we have checked the reactivity of reagents with proteins by analyzing all reagents half and double concentrations within first minutes of incubation. The obtained results are shown in (Figure **3**) after FI correction for the background signal. In all assays, we had linear increase in FI, corresponding to the increase of protein concentrations in samples. For all measurements R^2 was more than 97% which indicates accuracy of our assays and reliability of the procedures used in our study.

By combining these results, no differences in FI by assay with NanoOrange as non-amine reactive dye which was indicative for total protein amount in all tubes with trypsin and controls. However, significant increase in FI by incubation with trypsin is reflecting protein cleavage and increasing number of N-termini that have reacted with fluorogenic compounds when assayed by Chromeo P503 or CBQCA kit as amine reactive dyes.

Protein assay methods using highly sensitive amine reactive reagents represent reliable approach for analyzing proteolysed samples. Based on previously published works, amine reactive spectrophotometric reagents and CBQCA kit were shown useful for estimating increased protein fragmentation upon digestion [17, 18, 50]. In this study, we have shown that pyrylium based fluorogenic amine reactive dyes such as Chromeo P503 may be equally efficient in analyzing samples of proteolytic degradation by trypsin. Further studies are needed for full assessment of sensitivity, accuracy and reliability of these reagents in variety of biological samples.

CONCLUSION

The obtained data demonstrated the utility of assays based on detection of free N-terminal amino group in analyzing products of protein degradation by trypsin. Protein assays using NanoOrange kit, a non-reactive amine dye, showed no significant differences in FI among samples containing trypsin and controls. On the other hand, samples analyzed by Chromeo P503 and CBQCA, as amine reactive fluorogenic reagents, clearly showed correlation between FI and trypsin activity in protein samples.

LIST OF ABBREVIATIONS

BSA = Bovine Serum Albumin

FI = Fluorescence Intensity

CBQCA = 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde

Py = Pyrylium

PBS = Phosphate Buffer Saline

BCB = Bicarbonate Carbonate Buffer

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This work was supported by the Deanship of Scientific Research of The University of Jordan, grant number 156-9/2010.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank Deanship of Scientific Research of The University of Jordan for their financial support. In addition, we would like also to thank Mr. Mohammad Salah Abdullah and Prof. Mamoun Ahram from Cellular Biochemistry Laboratory for their kind help.

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