



A simple method to determine trypsin and chymotrypsin inhibitory activity

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Abstract

A colorimetric method for serine protease inhibition was modified using *N*-Acetyl-DL-Phenylalanine β -Naphthylester (APNE) as the substrate and *o*-Dianisidine tetrazotized (oD) as the dye. The reaction generated a single peak absorbing at 530 nm for both trypsin and chymotrypsin. Standard curves with increasing enzyme concentrations showed strong linearity. A standard curve for the serine protease inhibitor, Bowman–Birk Inhibitor (BBI), has been made using this modified method. The IC_{50} for 3 U of trypsin was found to be 33 ng and the IC_{50} obtained for 3 mU of chymotrypsin was 53 ng. A recombinant BBI (rBBI) gene was constructed, cloned and expressed in the yeast *Pichia pastoris*. Evaluating samples of rBBI for protease inhibitory activity by the gel activity method failed to quantify the inhibitor amounts, due to high sensitivity for trypsin inhibition and low sensitivity for chymotrypsin inhibition. After development, the results could not be quantified, even to the extent that 1 μ l of rBBI could not be detected with chymotrypsin inhibition. Therefore, a modified method for trypsin and chymotrypsin inhibition was used to evaluate the level of rBBI-expression for these same samples. The level of rBBI expression was calculated to be 50–56 ng/ μ l of media. These amounts fit into the range of values previously obtained by Western blot analysis. This modified method allows us to combine the sensitivity of the gel activity method with the quantification attributes of a Western blot. Thus, the modified method represents a significant improvement in speed, sensitivity and reproducibility over the gel activity method.

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1. Introduction

The Bowman–Birk Inhibitor (BBI) belongs to the serine-protease inhibitor family, harboring a unique function of independently inhibiting trypsin and chymotrypsin [1]. There is a growing interest in BBI and BBI-like molecules among the medical community, due to their anti-cancer activity [2–8]. BBI was initially isolated from soybean and was later found in other plants such as rice, potatoes and beans [1,7,9,10]. Several methods to measure the inhibitory activities of BBI and related protease inhibitors have been developed during the last 50 years [9,11–15]. In order to simplify the production of BBI, its gene was constructed and expressed in the yeast *Pichia pastoris* expression system [16]. In an attempt to assess the level of BBI expression and secretion in this expression system, a sample of the growing media was tested for protease inhibitory activity. It was found that the standard method to evaluate trypsin activity, using Tosyl-L-arginins methyl ester (TAME) as a substrate, failed due to the media inhibition of trypsin activity [13,16]. As a result, Hetzroni (1997) adapted a method that was developed for the characterization of protease inhibitor profiles from potatoes, barley and legume using protease-inhibitor SDS-PAGE activity gels [9,17,18]. This method can qualify the activity of the protease inhibitors but not the quantities of expressed protein. In this article, we describe an improved method that yields fast, reliable, sensitive, reproducible and quantifiable results, which can quantify the inhibition activity directly from the yeast growing media.

2. Materials and methods

2.1. Chemicals

The substrate *N*-Acetyl-DL-Phenylalanine β -Naphthylester (APNE), the blue dye *o*-Dianisidine tetrazotized (oD), and Bowman–Birk trypsin–chymotrypsin Inhibitor (BBI) were purchased from Sigma-Aldrich (St. Louis, MO, USA); *N,N*-dimethylformamide (DMF), and Acetic acid were purchased from Acros Organics (Morris Plains, NJ, USA).

2.2. Enzymes

Trypsin, with a specific activity of 50,500 U/ml, and α -chymotrypsin (EC 3.4.21.1) with a specific activity of 51 U/mg protein, were purchased from Sigma-Aldrich. Trypsin was used at a dilution of 3–5 U/ml, and chymotrypsin was used at 3–5 mU/ml. Dilutions were made with 50 mM citric acid buffer at pH 3 and 5 for trypsin and chymotrypsin, respectively, except for the last dilution (to 0.5 U/ml or 0.5 mU/ml), which was made in 50 mM Tris–HCl pH 8.0. All experiments began from the latter enzyme-dilutions. One unit of chymotrypsin will hydrolyze 1.0 μ mol of *N*-benzoyl-L-Tyrosine ethyl ester (BTEE) per minute at pH 7.8 at 25 °C. One unit of trypsin will hydrolyze 1.0 μ mol of *N*-alpha-benzoyl-L-Arginine ethyl ester (BAEE) per minute at pH 7.6 at 25 °C.

2.3. Gene construction and expression

All primers were ordered from the Integrated DNA Technologies (Coralville, IA, USA). BBI was synthesized based on the native BBI sequence from *Glycine max* (GeneBank, accession number X68704). Synthesis of the genes was similar to what was published in the past with some changes [16]. BBI-1; 5'ggatcctcgcgatgatgagtcttcaaaaccatgctgtgatcaatgcgcatgcacaaagtcaaac cctct 3', BBI-2; 5'agctgaatggcacgaattcagctctcatatctgaacagcggcattgagagggtttgactttgtgc atgcgcattga 3' BBI-3; 5'agatatgagactgaattcgtgccattcagctttaaactgtgtat-ttgcattatcgtatcc tgcacagtgttttgtgtgacataaccgatttc 3', BBI-4; 5'ctgcagagctctagagctgactt-agtcttctgtcatcctc actgggcttgcaggttcatagcagaaaatcggttatgtcaacacaaaaaacctg 3' BBI-5; 5'ggatcctcgcgatgat gagtc 3' and BBI-6; 5'ctgcagagctctagagctgac 3'. All reactions were carried out with Pfu polymerase (Stratagene, La Jolla, CA), using the following cycles: 95 °C for 10 min; 7 cycles of 95 °C 1 min, 50 °C 1 min, and 72 °C for 1 min; 72 °C for 10 min, to generate the two fragments. The same cycles were used for gene amplification (30 cycles) in a Lab-Line PCR instrument (Lab-Line Instruments, Melrose Park, IL, USA). BBI transformation, screening and expression were carried out in the *P. pastoris* expression kit as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Colonies were selected for His⁺ Mut^s. Yeast was grown in Buffered Glycerol-complex Medium (BMGY) and expression was induced in a Buffered Methanol-complex Medium (BMMY) containing 1% Methanol. BBI inhibitory activity was measured directly from the media 3 days after induction.

2.4. Substrate preparation and reaction

Substrate and dye mixture were prepared just before the measurements being taken. Trypsin was kept in dilutions in 50 mM citrate buffer (pH 3) and chymotrypsin was kept in 50 mM citrate buffer (pH 5), environments at which the enzymes were found to be most stable [19]. The reaction mixture was composed of 10% DMF and 50 mM Tris–HCl pH 8.0. APNE was dissolved in DMF to a final concentration of 0.75 mM, thereafter; oD was dissolved in Tris–HCl 50 mM pH 8.0 to a final concentration of 1 mM. Both solutions were combined and vortexed before use. At all times, the dye and thereafter the mixture, were protected from the light. The reaction began when the substrate–dye mixture was added to the plastic cuvette (Fisher Scientific, Morris Plains, NJ, USA) containing trypsin or chymotrypsin either with or without the inhibitor. All measurements were done in triplicate at 25 °C. The triplicate inhibition values differed by no more than 10%. The reaction was followed for 3 min under a Beckman DU 640 Spectrophotometer (Somerset, NJ, USA) at a wavelength of 530 nm. Presented results are the residual activities from the control expressed in percent. Protease-inhibition activity gels were carried out as shown previously [9].

3. Results and discussion

3.1. Wavelength scans of trypsin and chymotrypsin products

It was previously found that the growth media of yeast contains inhibitory activity against the trypsin substrate, Tosyl-L-arginine methyl ester (TAME) [16,20]. Hence,

another method needed to be adapted for the evaluation of the recombinant BBI (rBBI) inhibitory activity expressed in yeast. There are various methods to measure protease activity, some use substrates that their reaction products absorb at the UV spectrum such as: acetyl-L-tyrosine ethyl ester (ATEE) for chymotrypsin and BAEE, TAME for trypsin [16,19,20]. In order to follow the reaction progression, the formation of a unique product must be monitored. A wavelength scan of the reaction mixture produced a single peak at 530 nm (Fig. 1). This result was obtained separately with trypsin and chymotrypsin, using APNE as the substrate and oD as the dye. The absorbance spectrum was similar for both trypsin and chymotrypsin activity-products (Fig. 1A,B). Initially, the reaction was terminated by the addition of 1% acetic acid, with the absorbance profile remaining similar for both trypsin and chymotrypsin. In addition, the spectrum did not change as the reaction progressed when different units of enzyme were added to the reaction (data not shown). Since the product was best detected at 530 nm without any interference, all

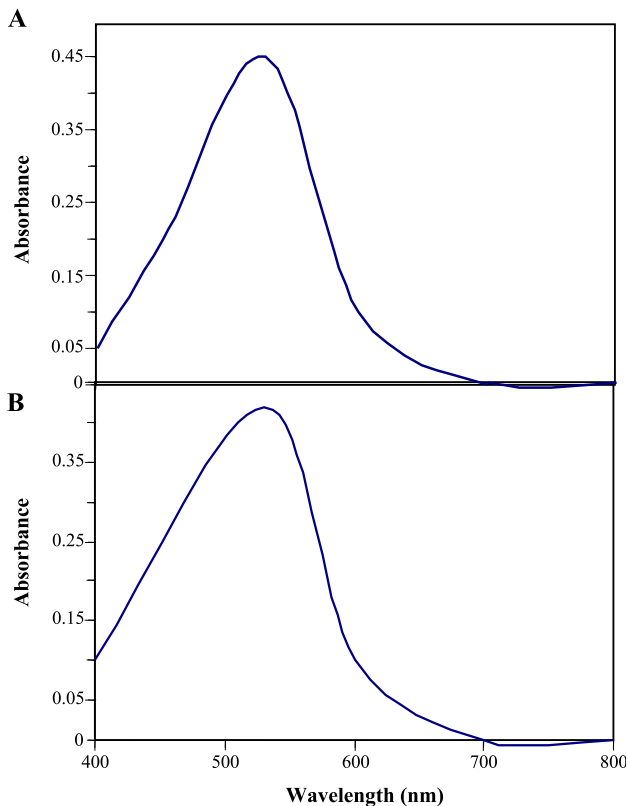


Fig. 1. Wavelength scan for the proteolysis product of the reaction mixture containing trypsin (A) and chymotrypsin (B) using 0.75 mM APNE and 1 mM oD as the dye final concentrations, in 10% DMF and Tris–HCl 50 mM pH 8.0. The reaction was carried out with 3 U of trypsin and 3 mU of chymotrypsin for 3 min. The reaction mixture was scanned between the wavelengths of 400 to 800 nm, a mixture with no enzyme was used as a blank. The results show the absorbance in relation to the wavelength.

reactions for both enzymes were monitored at this wavelength without the addition of acetic acid.

3.2. Trypsin and chymotrypsin activity and inhibition curves

In order to examine the number of enzyme-units needed for a linear progression of the reaction, the enzyme-activity was monitored at 530 nm with various concentrations of trypsin or chymotrypsin. The activity of trypsin was measured for a concentration range of 1 to 5 U/ml. Fig. 2A shows the regression line obtained after 3 min of reaction incubation. The calculated linear formula was $y=0.119X-0.0244$ with $R^2=0.976$. Activity of chymotrypsin was measured for a concentration range of 1 to 5 mU/ml. Fig. 2B shows the regression line obtained after 3 min of activity. The calculated linear formula $y=0.134X+0.004$ with $R^2=0.995$. Enzyme working dilutions selected for this method were in the range allowing a change of approximately 0.5 O.D unit at 530 nm in 3 min (Fig. 2). Thus, the number of enzyme-units used for each reaction were 3 and 5 U of trypsin, and 3 and 5 mU of chymotrypsin. Enzyme working dilutions in some other methods were found to be of 4–5 units per reaction with different substrates than APNE [12,13,19]. Also, in the protease gel activity method [9], an excess amount of enzymes were used, 202 U/ml of trypsin and 5.1 U/ml of chymotrypsin, in comparison to the modified method presented in this paper. In order to obtain a standard curve for the

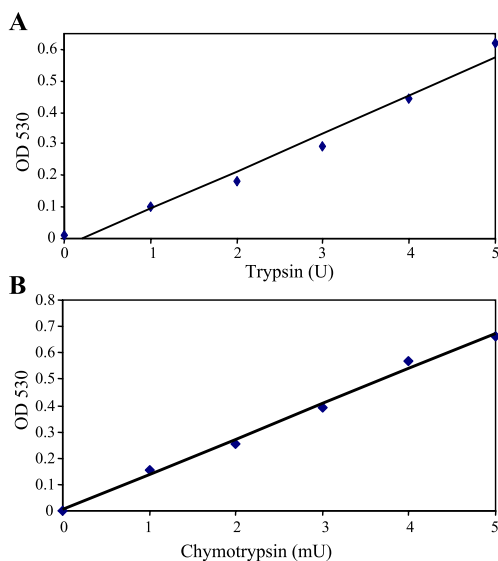


Fig. 2. Standard curve for the product received from the reaction containing trypsin (A) and chymotrypsin (B) using 0.75 mM APNE and 1 mM oD as the dye final concentrations, in 10% DMF and Tris–HCl 50 mM pH 8.0. The reaction was performed with 1 to 5 U of trypsin and 1 to 5 mU of chymotrypsin for 3 min. The reading was carried out in a spectrophotometer at 530 nm. The results show the regression line of absorbance as a function of the increased amounts of enzyme. Each point is a mean of the triplicate measurements with variation of no more than 10%.

inhibitory activities of BBI for trypsin and chymotrypsin, a range of BBI concentrations was added to the reaction mixtures. Fig. 3A shows the trypsin (3 U) inhibition curve at different concentrations of BBI. The calculated IC_{50} of BBI in this reaction was found to be 33 ng. The inhibition was linear with $R^2=0.961$. Fig. 3B shows the chymotrypsin (3 mU) inhibition curve at different concentrations of BBI. The calculated IC_{50} of BBI in this reaction was 53 ng. The inhibition was linear with $R^2=0.990$. IC_{50} for BBI inhibition for 5 U of trypsin and 5 mU of chymotrypsin was 55 ng and 100 ng, respectively (data not shown). The ability to alter the enzyme concentration demonstrates the flexibility of this technique, enabling measurement at different ranges of inhibitor concentrations, and adjusting its sensitivity for low or high amounts of inhibitors.

3.3. Gene construction

BBI was synthesized based on the native BBI sequence from *G. max* (GeneBank, accession number X68704). The gene was constructed by a two-step polymerase chain reaction (PCR) using 6 oligomers. First, BBI-1 as the forward primer and BBI-2 as the reverse primer were used to generate 115 bp from the 5' end of the gene.

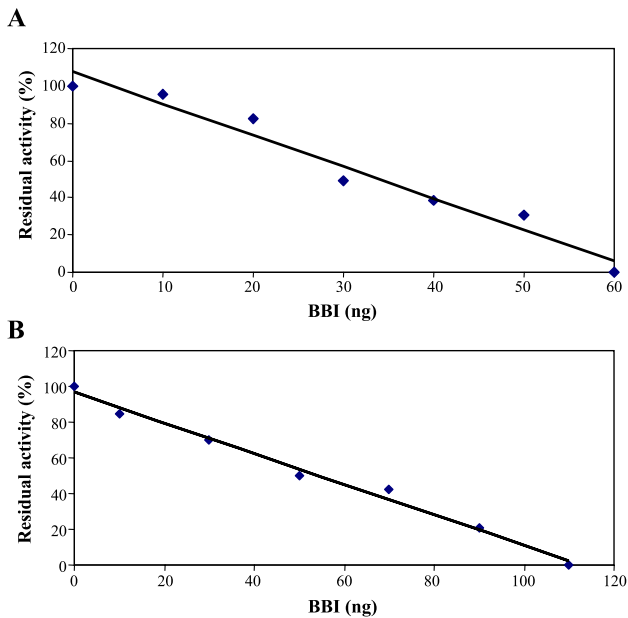


Fig. 3. Effect of different concentrations of BBI on trypsin (A) and chymotrypsin (B) activities. The reaction mixture contained 0.75 mM APNE and 1 mM oD in 10% DMF and Tris-HCl 50 mM pH 8.0 and 3 U of trypsin or 3 mU of chymotrypsin. The product was measured after 3 min at 530 nm. The results show the regression line of absorbance as a function of BBI concentration. Each point is a mean of a triplicate measurement with variation of no more than 10%. Residual activity is calculated as the ratio between the measurements of enzyme-activity product accepted from the reaction mixture containing BBI to the measurement of product from the reaction mixture without BBI and presented in percent.

Second, BBI-3 as the forward primer and BBI-4 as the reverse primer were used to generate a 162 bp from the 3' end of the gene (Fig. 4I, II). The two generated fragments shared 30 bases on the 3' end of the first fragment and the 5' end of the second fragment. In order to generate the full length of the gene, the two fragments were joined and subjected to another round of PCR with two additional primers, BBI-5 as the forward primer and BBI-6 as the reverse primer (Fig. 4III). The final product, BBI gene (213 bp), was cloned into the cloning vector, pGEM-7Z (Promega, Madison, WI, USA). Thereafter, BBI was sub-cloned into the yeast expression vector, pPIC-9 (Invitrogen).

3.4. Level of BBI expression

The yeast system of *P. pastoris* was used to express the recombinant BBI (rBBI) gene. In order to simplify the isolation of rBBI, the vector was designed to direct the expressed protein into the growth media. In previous experiments, before we developed this technique, the levels of rBBI expression were 20–40 ng/ μ l, depending on the specific experiment. Calculations were done by Western blot analysis using anti-BBI antibodies raised in rabbits and normalized by a known amount of BBI purchased from Sigma (Yakoby and Raskin, unpublished). This method allowed us only to quantify rBBI levels but not its activity. As mentioned previously, the growth media of yeast contains inhibitory activity against the trypsin substrate, TAME [16,20], thus, another method was necessary for the evaluation of the rBBI inhibitory activity, directly from the yeast growing media. The method chosen was initially developed for the characterization of protease inhibitors profiles in potatoes [18], barley [17] and later

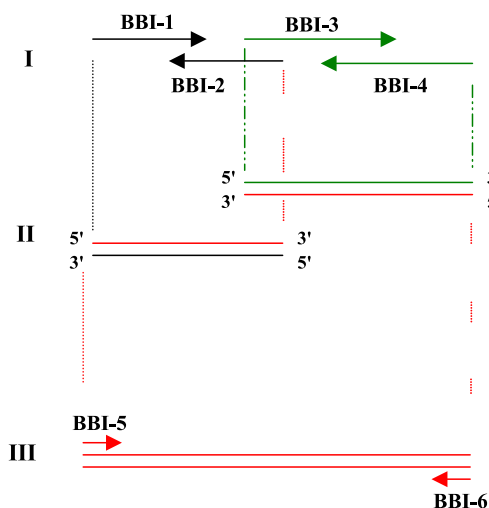


Fig. 4. Diagram of rBBI gene construction using six primers. The construction was carried out in two steps. The first step used BBI-1, 2 to generate half the gene while the other half was constructed with the primers BBI-3, 4 (part I), in order to obtain the two fragment of the gene (part II). In the second step, the two fragments were joined and amplified by using the primers BBI-5, 6 (part III). All reactions were carried out in a PCR machine.

adapted for legume seeds [9], using protease-inhibitor SDS-PAGE activity gels [9,17,18]. Samples of rBBI, expressed in *P. pastoris*, were tested in the activity-gel method. Fig. 5 shows samples of media with expressed rBBI, separated on SDS-PAGE and assayed for the activity inhibition of trypsin (A) and chymotrypsin (B). The 1, 5 and 10 μl /lane of the rBBI containing media, inhibited both trypsin and chymotrypsin. The gel activity method was less sensitive for chymotrypsin inhibition, and failed to detect 1 μl of the media sample containing rBBI. Moreover, it would be very difficult to accurately determine the amounts of rBBI in the gel activity assay based on the results (Fig. 5), due to the over-sensitivity to trypsin inhibition and the reduced sensitivity for chymotrypsin. The gel activity method was working with media taken directly from the growing-flask, but we could not adequately quantify rBBI protein levels; we modified this method to allow accurate quantification of the inhibitory activity.

The level of protein secretion was calculated based on the BBI inhibition curves for trypsin and chymotrypsin (Fig. 3), and compared to an aliquot of rBBI for its protease-inhibition-activity. The level of rBBI expression with both trypsin and chymotrypsin assays was 50–56 ng/ μl for this experiment (Fig. 6A,B). The amounts quantified with the current method are in the range of what was previously found using anti-BBI antibodies. In addition, these comparable results obtained with trypsin and chymotrypsin, further support the reproducibility and accuracy of this new method. The developed method has the potential to be adjusted for fluorometry, since the product absorbs at 530 nm, unlike other substrates with UV absorbance. Although no attempt to use a

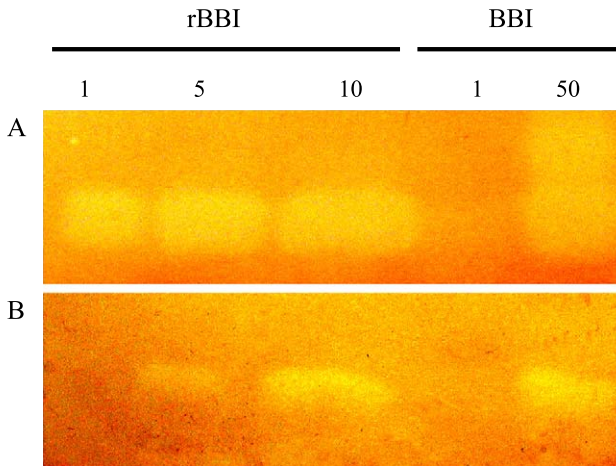


Fig. 5. SDS-PAGE activity gel developed for protease inhibitory activity of the different amounts of rBBI (1, 5, and 10 μl), and BBI (1 and 50 ng), loaded in each lane, on trypsin A and chymotrypsin B. rBBI and BBI samples were run in non-denaturated conditions in 15% SDS-PAGE. The gels were soaked in 2% Triton \times 100 for 30 min prior to any additional treatment. Thereafter, the gels were placed in a solution with trypsin (200 U/ml) or chymotrypsin (5 U/ml) for 10 min with a gentle agitation for 10 min at 37 °C. The gels were then washed five times with distilled water and put in a developing solution containing 0.75 mM APNE and 1 mM oD, in 10% DMF and Tris–HCl 50 mM pH 8.0, for 10 min at 37 °C. The development was terminated after 10 min of incubation with a solution of 3.5% acetic acid.

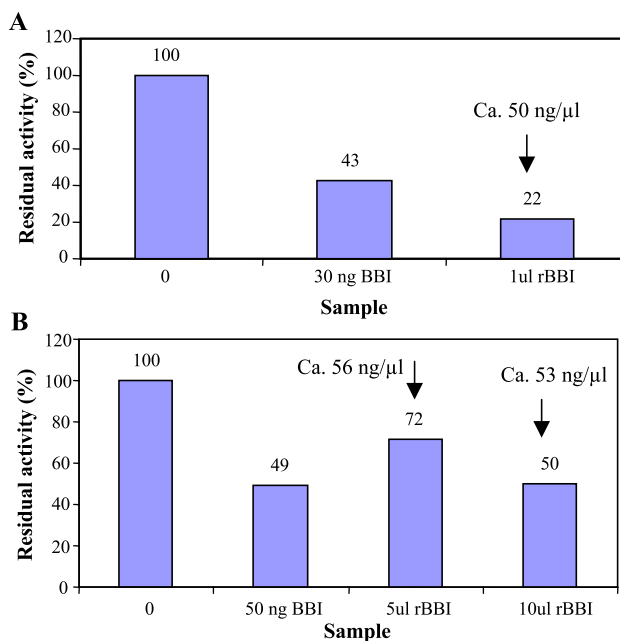


Fig. 6. Quantification of rBBI production by testing the recombinant yeast growing media directly for protease inhibitory activity of trypsin (A) and of chymotrypsin (B) using 0.75 mM APNE and 1 mM oD in 10% DMF and Tris–HCl 50 mM pH 8.0. The reaction contained 3 U of trypsin or 3 mU of chymotrypsin and measurement was taken after 3 min at 530 nm. The protease inhibitory activity was used to calculate the amount of expressed rBBI. The results show the residual activity of the enzymes in relation to different amounts of rBBI sample. Residual activity was calculated as the ratio between the measurements of enzyme-activity product accepted from the reaction mixture containing BBI to the measurement of product from the reaction mixture without BBI and presented in percent. The calculated (Ca.) amounts of rBBI were obtained from the standard curve of different BBI concentrations (Fig. 3) and are expressed in ng/μl. Each point is a mean of a triplicate measurement with variation of no more than 10%.

fluorometer was made, this adjustment may make the method more attractive for high throughput screening.

4. Conclusion

The technique developed for the determination of trypsin and chymotrypsin inhibitory activity by rBBI is simple, accurate, rapid, sensitive and reproducible in comparison to the protease-activity gel method that can give only qualitative measurements of the inhibitor activity and Western blot analysis that can only quantify rBBI protein levels. In addition, compared to other methods using TAME as substrate, the method we developed allows the assessment of protease inhibitory activity directly from the yeast *P. pastoris* expression media. This method has also the potential to be adjusted for fluorometry for high throughput screening. In addition, this method may also be adjusted for the detection of

other serine protease inhibitors, with activities against human diseases such as cancer and obesity, with fast and reliable results.

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