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An Improved End-Point Fluorimetric Procedure for the Determination of Low Amounts of Trypsin Activity in Biological Samples Using Rhodamine-110-Based Substrates

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Abstract A novel end-point fluorimetric procedure based on the use of rhodamine-110-labeled specific substrate was developed to determine trypsin activities in biological samples. We evaluated the ability of trichloroacetic acid and acetic acid to stop the enzymatic reaction without hindering the detection of the fluorescence of rhodamine-110 released into the reaction mixture from the specific substrate (CBZ-L-alanyl-L-arginine)$_2$-rhodamine-110. Trichloroacetic acid decreased markedly the fluorescence of rhodamine-110, even at low concentrations. On the other hand, the addition of 50 mmol/l acetic acid inactivated efficiently trypsin activity, causing minor effects on rhodamine-110 fluorescence. The proposed procedure was more sensitive than the spectrophotometric end-point method using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as substrate. The possibility of carrying out end-point fluorimetric assays improves the performance of monocell fluorimeters by setting specific conditions optimal for each enzyme activity independently of the fluorimeter. This method also allows replicate assays to be conducted simultaneously, resulting in considerable time saving and in increased performance of low-cost equipment.

Keywords End-point assay · Fluorimetric assay · Protease activity · Rhodamine-110 · Trypsin

Abbreviations

CBZ	Benzyloxycarbonyl
TCA	Trichloroacetic acid
Rho-110	Rhodamine-110
BPC	Phosphate-citrate buffer
RFU	Relative fluorescence units
BAPNA	*N*-α-Benzoyl-DL-arginine-*p*-nitroanilide

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Introduction

A wide variety of procedures have been developed to quantify the amount of proteolytic activity existing in biological samples, most of them based on spectrophotometric measurements of the products released from substrate proteins [1–5] and specific synthetic substrates [6–10]. However, spectrophotometric procedures are frequently not satisfactory, either because they cannot be used with crude tissue homogenates due to interferences in absorbance or because weak proteolytic activity is present in samples. Fluorimetric methods are then feasible alternatives to overcome such limitations because fluorogen-labeled substrates link specificity and sensitivity in a very convenient manner. Therefore, they are widely used in proteolytic assays [11–17]. Different models of fluorimeters are commercially available, from multi-sample to monocell apparatus; the latter ones, despite their lower cost, show several limitations such as (a) difficulties in keeping optimal reaction parameters required for enzyme activity and (b) only one measurement at a time can be carried out, this being time consuming when multiple samples are analyzed.

Most of fluorimetric procedures described for the quantification of protease activities are based on continuous monitoring of the reaction and further calculation of kinetic parameters. The replicates must be carried out one at a time if multi-plate fluorimeters are not available, becoming a time-consuming process and increasing the likelihood of experimental errors. However, in those studies in which the kinetic parameters of the enzyme reaction are not required, but only a final quantification is required, end-point procedures would be preferred. In this sense, a wide amount of end-point colorimetric procedures are available, but this contrasts with the lack of similar procedures based on fluorescent substrates.

Therefore, this work addresses the possibility of developing a simplified end-point fluorimetric assay for the detection and quantification of low amounts of trypsin activity. Efforts are focused to the identification of an agent that (1) is effective in stopping protease activity, (2) exerts minimal negative effects on the fluorescence of rhodamine-110 (Rho-110) released from a specific fluorimetric substrate, and (3) allows sensitive quantification of protease activity.

Experimental

Reagents

Two specific substrates for trypsin activity were used: *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), a colorimetric substrate purchased from Sigma Chem. Co. (St Louis, MO, USA), and the bis-peptide Rho-110 derivative $(CBZ\text{-}Ala\text{-}Arg)_2$-rhodamine-110, a fluorogenic substrate obtained from Molecular Probes, Inc. (Eugene, OR, USA). Trypsin from porcine pancreas (EC 3.4.21.4, Type IX, activity—14,900 units/mg protein) was used as commercial enzyme source (Sigma Chem. Co.).

Fluorimetric Trypsin Assay

The fluorimetric assay for trypsin activity was carried out as follows: 10 μl of trypsin dilutions ranging from 1 mg/ml to 1 pg/ml was mixed with 1.5 ml of borate–phosphate–citrate buffer (BPC buffer; 5.7 mmol/ml sodium tetraborate, 20 mmol/l disodium hydrogen phosphate, 13 mmol/l sodium citrate), pH 8.0, containing 1 μmol/l (CBZ-Ala-Arg)2-rhodamine-110 as

substrate. The reaction mixture was incubated for 1 h at 37 °C in a thermostatic water bath, and the fluorescence of the products released during the enzymatic reaction was measured using a monocell Versafluor™ fluorimeter (BioRad, Richmond, CA, USA) at 498 nm excitation and 521 nm emission wavelengths, according to the recommendations of the substrate manufacturer [18]. Blanks without enzyme were run simultaneously. Linearity of the proteolytic activity was previously confirmed against enzyme concentration and time. All the assays were performed at least in triplicate.

Effects of Trichloroacetic Acid and Acetic Acid on Fluorescence

Trichloroacetic acid (TCA) and acetic acid are reagents normally used with the purpose of inactivating protease activity in spectrophotometric assays [1–3, 5, 6, 8]. In order to assess the effect of these substances on the fluorescence of the products released in the reaction mixture, sufficient amount of Rho-110 from the Rho-110-labeled substrate was released by mixing 1.5 ml of BPC buffer, pH 8.0, 2.5 μl of 0.3 mmol/l trypsin fluorogenic substrate, and 5 μl of aqueous trypsin solution (1 mg/ml). The mixture was incubated at 37 °C until the substrate was completely hydrolyzed (fluorescence lecture did not increase any more, about 20 min). Aliquots were made, and then acetic acid and TCA were added in order to reach final concentrations of 1, 25, 50, 100, 250, 500, and 1,000 mmol/ml, keeping constant volumes. The solutions were vortexed and allowed to stand for 10 min, and then the remaining fluorescence (relative fluorescence units (RFU)) was measured. Results were expressed as percentage, considering 100% the fluorescence measured in the mixtures in which distilled water was added instead of acetic acid or TCA solutions.

Concentration of Acetic Acid Needed to Inactivate Enzyme Activity

A reaction mixture containing 5 μl of trypsin solution (1 mg/ml), 1.5 ml of BPC buffer, pH 8.0, and 0.1 mmol/ml Rho-110 derivative substrate was incubated at 37 °C. After 5 min, different amounts of acetic acid were added to the replicates, reaching final concentrations ranging from 10^{-3} to 1 mol/l. Values of RFU were recorded immediately after acetic acid was added (this point was considered fluorescence at time 0), and the incubation was further continued under the identical conditions for 1 h. Fluorescence was measured again after 15, 30, 45, and 60 min in order to assess if the reaction was completely stopped (fluorescence did not increase compared to that measured immediately after the addition of acetic acid). TCA was not assayed due to its marked negative effect on the fluorescence of Rho-110 resulting from the previous experiment.

Sensitivity

Given that the main advantage of fluorimetric procedures compared to colorimetric assays is their much higher sensitivity, trypsin activity was also measured according to the spectrophotometric procedure described by Erlanger et al. [6] in order to compare the proposed end-point fluorimetric assay against the colorimetric method. Trypsin dilutions ranging from 1 mg/ml to 1 pg/ml were used; 10 μl of each dilution was added to 1.5 ml of 1 mmol/l BAPNA prepared in BPC buffer, pH 8.0 (final enzyme amounts ranging from 10 μg to 0.01 pg per tube). The reaction mixture was incubated (60 min, 37 °C) in a thermostatically controlled water bath and then stopped by adding 0.2 ml of 30% (*v*/*v*) acetic acid. The amount of *p*-nitroanilide released was measured spectrophotometrically at 410 nm. Blanks without enzyme were run simultaneously. All the assays were carried out at least in triplicate.

Statistical Analysis

Data were analyzed by one-way ANOVA followed by a comparison of means (Tukey's test). Because values were expressed as percentage, they were normalized using the arc sine transformation of their square root [19] prior to statistical analysis. All statistics were conducted using the Statgraphics Plus 4.0 specific software (Statistical Graphics Corp., Rockville, MA, USA).

Results and Discussion

Effects of TCA and Acetic Acid on Rhodamine-110 Fluorescence

The effects of increasing amounts of both reagents on the fluorescence of Rho-110 released from $(CBZ\text{-}Ala\text{-}Arg)_2$-rhodamine-110-specific substrate are shown in Fig. 1. It is significant that TCA decreased fluorescence in a higher extent than acetic acid, independently of the concentration considered. Fluorescence dropped 11.9% when 25 mmol/l TCA was added. In contrast, acetic acid caused only 1.1% decrease at such concentration. At the highest concentration assayed (1 mol/l), acetic acid and TCA were responsible for 12.2% and 37.6% decrease in RFU, respectively. Differences between the effects caused by both substances were significant ($P<0.001$) at any concentration assayed.

The proposed end-point procedure is based on two premises: (1) the need of a substance that effectively inactive the enzymes present in the reaction mixture and (2) the minimum decline in Rho-110 fluorescence released from the substrate due to such substance. For a given amount of substrate and enzyme activity, the smaller the RFU value, the lower the sensitivity of the method. This might lead to under-estimation of trypsin activity existing in samples. The results found here indicate that TCA is an unsuitable reagent for such purpose; in contrast, acetic acid seems to meet the requirements related to low effect on Rho-110 fluorescence.

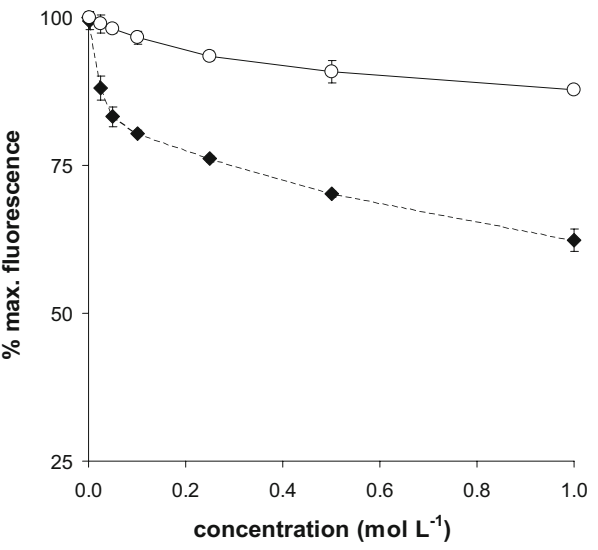

Fig. 1 Effect of increasing concentrations of acetic acid (*circles*) and trichloroacetic acid (*diamonds*) on the fluorescence of rhodamine-110 released from $(CBZ\text{-}Ala\text{-}Arg)_2$-rhodamine-110. Where not shown, standard error bars are *within symbols*

Concentration of Acetic Acid Required to Stop the Enzyme Reaction

In view of the fact that acetic acid affected the fluorescence of the reaction mixture in a lesser extent than TCA, the next stage was the assessment of the concentration needed to effectively stop the activity of the commercial trypsin. Under that perspective, increasing amounts of acetic acid were added to reaction mixtures containing a given amount of trypsin, as explained in the "Experimental" section, and the results obtained are shown in Fig. 2. Concentrations below 0.1 mol/l acetic acid were not able to inactivate trypsin activity, as evidenced by the continuous increase in fluorescence the next hour after acetic acid was added to the reaction mixture (differences in fluorescence between time 0 and 60 min were significant, $P<0.05$). The enzyme activity was completely inactivated when concentrations over 0.25 mol/l were used since no significant ($P<0.001$) residual increasing of RFU was observed after 60 min. According to these results, 0.5 mol/l acetic acid was considered the concentration effective in order to stop enzyme activity of commercial trypsin.

The convenience of an end-point fluorimetric procedure has been broached above; however, this approach has being fulfilled in literature only by the design of an end-point assay in which specific protease inhibitors were added to stop the reactions [20]. Nevertheless, the main limitation is that specific inhibitors are not commercially available for all the enzyme activities susceptible of using rhodamine-110-based substrates. Thus, the possibility of using an unspecific inactivating agent, as acetic acid, represents a clear advantage.

Sensitivity of the Procedure

Although the effect of acetic acid on Rho-110 fluorescence was lower than that observed for TCA (Fig. 1), nevertheless, a certain amount of fluorescence (9.2%) was lost when 0.5 mol/l acetic acid was added (Fig. 1). Thus, reasonable doubts appeared concerning the sensitivity of the proposed fluorimetric procedure compared to the colorimetric assay for trypsin (based on *p*-nitroanilide-labeled substrates; Erlanger et al. [6]). The contrast of both procedures was carried out according to the procedures already described in the

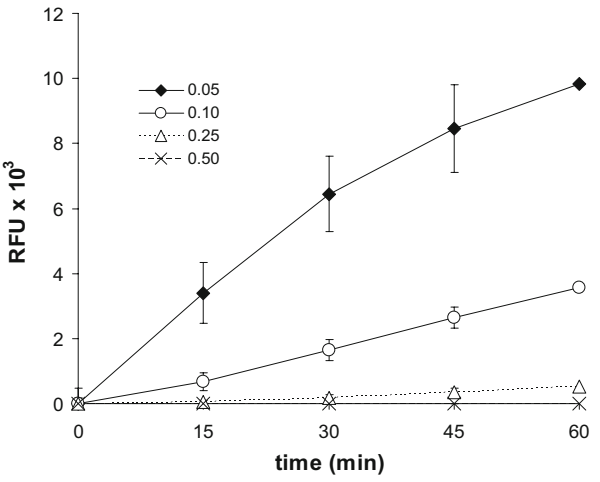

Fig. 2 Effect of the concentration (mole per liter) of acetic acid on residual trypsin activity. Enzyme activity was measured considering time 0 and RFU 0 those immediately after the addition of the different concentrations of acetic acid. Where not shown, standard error bars are within symbols. Concentrations below 0.05 mol/l (unable to inactivate trypsin) and over 0.5 mol/l (complete inactivation) are not shown for clarity of presentation

"Experimental" section, and the results are shown in Fig. 3. The detection limit of the spectrophotometric procedure under the conditions assayed here was approximately 100 ng of trypsin. On the other hand, the minimum amount of trypsin noticeable by the fluorimetric procedure was 10 pg of enzyme (sensitivity 10^4-fold higher in relative terms). In absolute terms, a variation of 5 ng in trypsin concentration caused a change of 0.005 in Abs 410 nm (due to *p*-nitroanilide released from BAPNA), whereas identical amount of enzyme increased fluorescence by 117.6×10^3 RFU (due to Rho-110 released from $(CBZ\text{-}Ala\text{-}Arg)_2$-rhodamine-110). Thus, even assuming that acetic acid decreased fluorescence of Rho-110 to a certain extent at the concentration that effectively inactivated trypsin (9.2% with 0.5 mol/l, Fig. 2), the sensitivity of the proposed end-point procedure is definitely kept much higher than the colorimetric end-point assay.

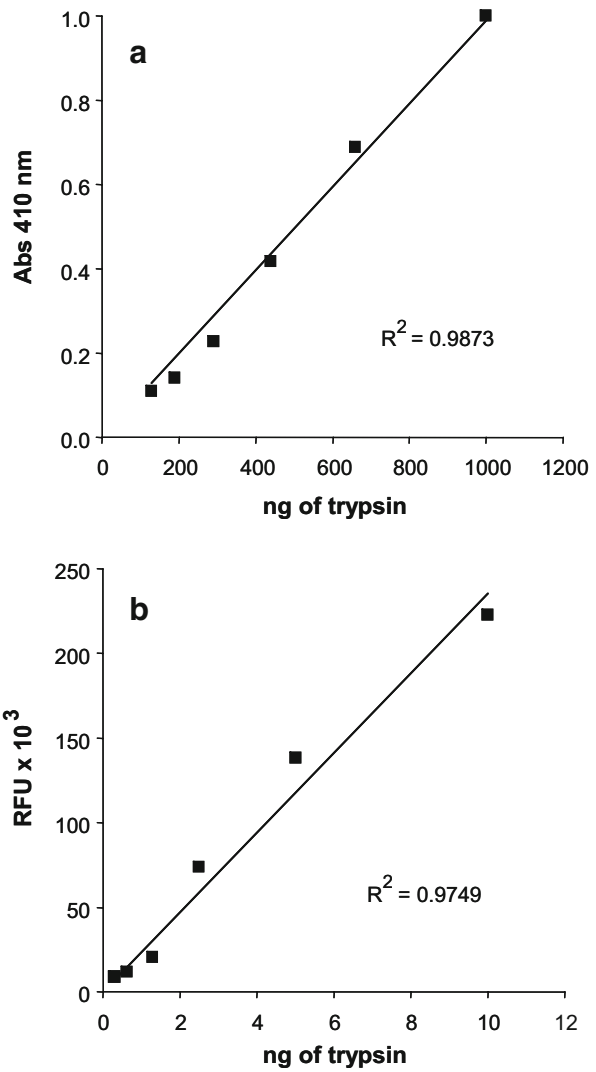

Fig. 3 Correlation between the amount of trypsin and Abs 410 nm (**a**, spectrophotometric assay) or RFU (**b**, fluorimetric assay). Notice the differences in the detection limits indicated by the amount of trypsin in *X* axis

Conclusion

The measurement of kinetic parameters in continuous reactions is necessary to characterize the catalytic activity of proteases. However, when the aim of a given study is merely the quantification of total amounts of enzyme activity existing in biological samples, the assessment of kinetic parameters in continuous assays is time consuming, especially when monocell fluorimeters are used, and then end-point methods are desirable. Moreover, monocell fluorimeters usually do not allow an accurate control of the reaction parameters affecting the enzyme catalysis, this leading to poor replication. The proposed end-point procedure overcomes such limitation, allowing the enzyme reaction to be carried out in an external bath of incubator, fulfilling the specific requirements optimal for each protease activity. Hence, multiple replications can be performed simultaneously, this resulting in considerable time saving and increased performance of low-cost equipment.

The effect of acetic acid was tested on Rho-110 fluorescence released into the reaction mixture, instead than on the trypsin-specific substrate itself. This means that the procedure proposed can likely be extended to any enzyme activity in which measurement is based on the use of Rho-110-labeled substrates and not merely to trypsin activity. The unspecific inactivation caused by acetic acid, together with the slight effects on fluorescence due to Rho-110, suggests the possibility of a wider use of the proposed procedure.

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References

1. Anson, M. (1938). The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *The Journal of General Physiology*, *22*, 79–89. doi:10.1085/jgp.22.1.79.
2. Walter, H. E. (1984). Proteinases: Methods with haemoglobin, casein and azocoll as substrates. In H. U. Bergmeyer (Ed.), *Meth enzym anal* (vol. 5, pp. 270–277). Weinheim, Germany: Verlag Chemie.
3. Tomarelli, R. M., Charney, J., & Harding, M. L. (1949). The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. *The Journal of Laboratory and Clinical Medicine*, *34*, 428–433.
4. Chavira Jr., R., Burnett, T. J., & Hageman, J. H. (1984). Assaying proteinases with azocoll. *Analytical Biochemistry*, *136*, 446–450. doi:10.1016/0003-2697(84)90242-2.
5. Sarath, G., de la Motte, R. S., & Wagner, F. W. (1989). Protease assays. In R. J. Beynon, & J. S. Bond (Eds.), *Proteolytic enzymes, a practical approach* (pp. 25–55). Oxford, UK: IRL Press.
6. Erlanger, B., Kokowsky, N., & Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics*, *95*, 271–278. doi:10.1016/0003-9861(61)90145-X.
7. Bieth, J. G., Spiess, B., & Wermuth, C. G. (1974). Synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochemical Medicine*, *11*, 350–357. doi:10.1016/0006-2944(74)90134-3.
8. Del Mar, E. G., Largman, C., Brodrick, J. W., & Geokas, M. C. (1979). A sensitive new substrate for chymotrypsin. *Analytical Biochemistry*, *99*, 316–320. doi:10.1016/S0003-2697(79)80013-5.
9. Gravett, P. S., Viljoen, C. C., & Oosthuizen, M. M. (1991). A steady-state kinetic analysis of the reaction between arginine esterase E-I from *Bitis gabonica* venom and synthetic arginine substrates and the influence of pH, temperature and solvent deuterium isotope. *The International Journal of Biochemistry*, *23*, 1085–1999. doi:10.1016/0020-711X(91)90149-H.
10. Gaertner, H. F., & Puigserver, A. J. (1992). Increased activity and stability of poly(ethylene glycol)-modified trypsin. *Enzyme and Microbial Technology*, *14*, 150–155. doi:10.1016/0141-0229(92)90174-M.
11. Smith, G. P., MacGregor, R. R., & Peters, T. J. (1982). Localization of leucine aminopeptidase and vitamin B-12 binding protein in rabbit peripheral blood polymorphonuclear leukocytes. *Biochimica et Biophysica Acta*, *719*, 532–538.
12. Smith, R. E., Bissell, E. R., Mitchell, A. R., & Pearson, K. W. (1980). Direct photometric or fluorometric assay of proteinases using substrates containing 7-amino-4-trifluoromethylcoumarin. *Thrombosis Research*, *17*, 393–402. doi:10.1016/0049-3848(80)90074-2.

13. Leytus, S. P., Patterson, W. L., & Mangel, W. F. (1983). New class of sensitive and selective fluorogenic substrates for serine proteinases. Amino acid and dipeptide derivatives of rhodamine. *Journal of Biochemistry*, *215*, 253–260.
14. Twining, S. S. (1984). Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical Biochemistry*, *143*, 30–34. doi:10.1016/0003-2697(84)90553-0.
15. Homer, K. A., & Beighton, D. (1990). Fluorometric determination of bacterial protease activity using fluorescein isothiocyanate-labeled proteins as substrates. *Analytical Biochemistry*, *191*, 133–137. doi:10.1016/0003-2697(90)90399-T.
16. Ueberschär, B., Pedersen, B. H., & Hjelmeland, K. (1992). Quantification of trypsin with radioimmunoassay in herring larvae (*Clupea harengus*) Compared with a highly sensitive fluorescence technique to determine tryptic enzyme activity. *Marine Biology (Berlin)*, *113*, 469–473. doi:10.1007/BF00349173.
17. Yasothornsrikul, S., & Hook, V. Y. H. (2000). Detection of proteolytic activity by fluorescent zymogram in-gel assays. *BioTechniques*, *28*, 1166–1173.
18. Haugland, R. P. (2000). *Handbook of fluorescent probes and research products* (9th ed.). Eugene, OR: Molecular Probes.
19. Sokal, R., & Rohlf, J. F. (1981). *Biometry: The principles and practice of statistics in biological research*. WH Freeman: San Francisco.
20. Grant, S. K., Sklar, J. G., & Cummings, R. T. (2002). Development of novel assays for proteolytic enzymes using rhodamine-based fluorogenic substrates. *Journal of Biomolecular Screening*, *7*, 531–540. doi:10.1177/1087057102238627.