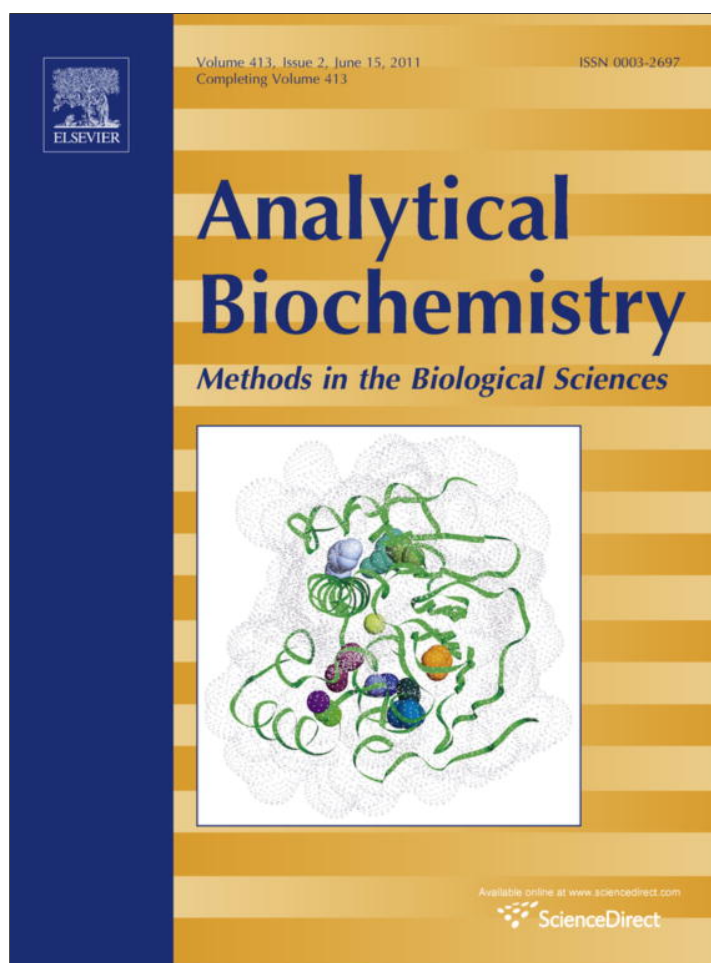


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Notes & Tips

NanoDrop fluorometry adopted for microassays of proteasomal enzyme activities

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ABSTRACT

NanoDrop spectrophotometry and NanoDrop fluorospectrometry are used almost exclusively to determine the concentrations of nucleic acids and proteins. We propose that NanoDrop fluorospectrometry can also be applied for measuring enzyme activities using fluorogenic substrates such as the proteolytic activities of the 26S proteasome. Because the NanoDrop ND-3300 device requires only 2 μ l of sample, the amount of sample extract, substrate, and cofactors used for an enzyme assay can be significantly reduced. In this report, we present exemplary microassays for proteasomal activities (chymotrypsin-, trypsin-, and PGPH [peptidyl-glutamyl peptide hydrolase]-like sites) in extracts of isolated hemocytes from a marine crab, *Cancer pagurus* (Crustacea).

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The use of highly sensitive fluorogenic substrates is becoming increasingly relevant not only in analytical and clinical enzymology but also in various other fields of life sciences, including biochemical, physiological, and biomedical research. Particularly important applications include the design of artificial substrates for use as a tool in cytology and pathology [1,2]. In this respect, the discovery of the intracellular ubiquitin-mediated proteolytic pathway and the controlled degradation of waste proteins by the multi-enzyme complex called 26S proteasome [3,4] has opened another important field for fluorometric enzyme assays [5–7].

Fluorogenic substrates as well as enzyme effectors may be quite expensive or available in limited amounts; the latter is particularly relevant if their isolation from natural sources is laborious or they are synthesized in small amounts for specific applications. Therefore, it would be ideal if the amount of substrate used per assay could be decreased through a reduction in assay volume. This may lead to a better scientific exploration of rare drugs. Similarly, reducing the assay volume may also allow enzymatic analysis within small medical biopsy samples, cell culture suspensions, and very small organisms and the parts thereof [8,9].

In our laboratory, we have developed NanoDrop fluorometry for routine measurements of enzyme activities within small samples. For example, activities of digestive enzymes have been measured in small marine invertebrates, and proteolytic enzymes have been measured in the muscle tissues from individual lobster larvae. In this report, we describe in detail a method for measuring 26S proteasomal activities in small amounts of hemocyte extracts from crustaceans. We also demonstrate the sensitivity of this method and describe its potential applications within various fields of the life sciences.

Because the NanoDrop device (ND-3300, version 2.7.0, PEQLAB Biotechnologie) requires only 1–2 μ l of reaction mixture for fluorescent measurement [10], the total volume of the assay was reduced significantly.

The reproducibility and linearity of the fluorescence signals were determined with the fluorophore 7-amino-4-methyl coumarin (AMC,¹ Fluka, product no. 08440). This fluorophore has excitation and emission wavelengths of 365 and 437 nm, respectively. A standard curve was prepared using solutions of AMC ranging in concentration from 0 to 20 μ M. These solutions were made from stock fluorophore dissolved in dimethyl sulfoxide (DMSO), which was then diluted in 26S proteasome assay buffer (50 mM Tris-HCl, 40 mM KCl, 5 mM MgCl₂, and 2 mM ATP, pH 8.0). The fluorescence of these standards was measured concurrently using the NanoDrop device and a conventional spectrofluorometer (Kontron SFM 25). The NanoDrop device requires 2 μ l of solution, whereas the conventional spectrofluorometer requires 1 ml of solution in semi-microcuvettes.

Proteasomal activities were assayed in hemocytes isolated from the hemolymph of the marine crab *Cancer pagurus*. Using a 1-ml sterile syringe with a 22-gauge needle, 50–100 μ l of hemolymph was collected by puncturing the intersegmental membrane at the base of a walking leg. To prevent coagulation, the hemolymph was mixed in the syringe with at least half a volume of anticoagulant solution (300 mM NaCl, 10 mM KCl, 10 mM HEPES, and 10 mM ethylenediaminetetraacetic acid [EDTA], pH 7.3). After collection, the hemolymph samples were transferred into 1.5-ml reaction tubes and centrifuged for 10 min at 600g and 10 °C. The supernatants were then discarded, and the pellets containing the hemocytes were resuspended in 100 μ l of 26S proteasome assay

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E-mail address: reinhard.saborowski@awi.de (R. Saborowski).¹ Abbreviations used: AMC, 7-amino-4-methyl coumarin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; PGPH, peptidyl-glutamyl peptide hydrolase; RFU, relative fluorescence units.

buffer. The suspended hemocytes were homogenized manually in microtubes with a stainless steel micropestle. The homogenates were centrifuged at 9000g for 10 min at 4 °C, and the supernatants were transferred into new 1.5-ml reaction tubes and stored at –80 °C until further analysis of enzyme activity and protein content [11].

The proteasomal activities were determined by the hydrolysis of fluorogenic peptides [12]. Hydrolysis of Boc-Leu-Arg-Arg-MCA (PeptaNova, product no. 3140) represents trypsin-like activity, Suc-Leu-Leu-Val-Tyr-AMC (Enzo Life Sciences, product no. P-802) represents chymotrypsin-like activity, and Z-Leu-Leu-Glu-AMC (Enzo Life Sciences, product no. 9345) represents peptidyl-glutamyl peptide hydrolase (PGPH)-like activity. Epoxomicin, at concentrations between 2.5 and 100 μM, was used as a highly specific inhibitor of the proteasome in all three assays [13].

The total volume of the enzymatic reaction mixture was 25 μl. The enzyme assay consisted 17.5–20 μl of assay buffer, 2.5–5 μl of sample, 1.25 μl of substrate, and 1.25 μl of inhibitor. All substrates were prepared just prior to the measurement as 10- or 20-fold stock solutions dissolved in DMSO. The reactions were started by the addition of the substrate into reaction tubes already containing assay buffer and the samples that were adjusted for a few minutes to the subsequent incubation conditions. The tubes were immediately transferred to an Eppendorf thermomixer and incubated at 30 °C under permanent agitation (350 rpm). To determine the linearity of the enzymatic reaction, 2-μl aliquots of the reaction mixture were removed from the tubes every 6–7 min for up to 1 h and applied to the optical cell of the NanoDrop instrument for fluorescence measurement.

Endpoint measurements were done exactly after predetermined periods of, for example, 1 h. For each enzyme assay, substrate blanks were run in parallel to control the rate of autolysis of the substrate during incubation. These blank assays contained only buffer and the substrate, and no enzyme was added. Excitation and emission wavelengths were 365 and 437 nm, respectively.

The reproducibility of the measurements of AMC fluorescence with the NanoDrop device was excellent. The standard curve was linear ($r^2 = 0.999$) for AMC concentrations up to 20 μM, which corresponded to approximately 52,000 relative fluorescence units (RFU) (Fig. 1). The deviation of the measured data from the calcu-

lated standard curve amounted to 5% on average. It was also much lower in the higher concentration range than at the very low concentrations between 0.5 and 1 μM (Fig. 1).

The reproducibility of the standard measurements was in the same range as, or even better than, that obtained by conventional fluorometry in 1-ml microcuvettes. The latter measurements showed progressively decreasing fluorescence at higher AMC concentrations, probably due to quenching effects. Nevertheless, the correlation between the average values obtained by both methods gave a coefficient r^2 of 0.998. For the quantification of fluorescence data, we used the NanoDrop software, which is capable of applying various curve fits such as linear, cubic, and spline fits.

The measurements of proteasomal activities showed a linear increase of fluorescence over time for each of the three substrates (Fig. 2). Assays of PGPH-like activities that were inhibited with epoxomicin showed a significantly reduced, but also linear, increase of fluorescence due to remaining activity. No trypsin-like and almost no chymotrypsin-like activities remained after inhibition with epoxomicin. The reproducibility of the measurements was very good, with an overall average variation of less than 5%.

These results confirm the applicability of NanoDrop fluorometry for enzyme assays in very small samples and, thus, make this method useful for studying precious proteasome inhibitors or effectors [14–16]. A small amount of extract (50–100 μl) is more than sufficient to run all three proteasomal enzyme assays, including controls and effectors, and to quantify protein as well. Moreover, the method enables fluorogenic enzyme assays to be conducted on small individual animals, which might be important in environmental, ecophysiological, or ecotoxicological studies [8].

Despite the demonstrated advantages, this method also has some disadvantages. The NanoDrop method can measure only one sample at a time, and samples must be carefully applied so that the volume is consistent and bubbles are avoided. Nevertheless, a skilled laboratory worker will be able to finish one measurement within 30 s. Accordingly, this method is not applicable to large-scale screening, but it is very well-suited for the explorative screening of samples on a laboratory scale.

In conclusion, NanoDrop fluorometry is a reliable and reproducible method for the measurement of proteasomal activities within very small amounts of samples such as hemocytes. The significant

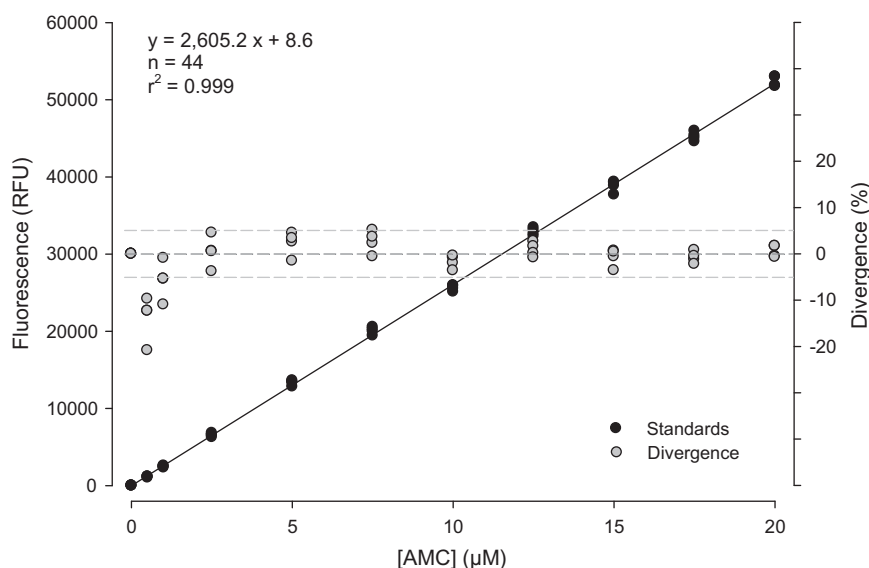


Fig. 1. Example of a standard curve for increasing AMC concentrations (0–20 μM, $n = 44$). The corresponding fluorescence, as detected by the NanoDrop ND-3300 device, is presented as relative fluorescence units. The residues of each measurement (gray circles) are included on the graph. The dotted gray lines indicate a range of $\pm 5\%$ divergence. The measurements scattered by less than $\pm 5\%$ in the range between 2.5 and 20 μM.

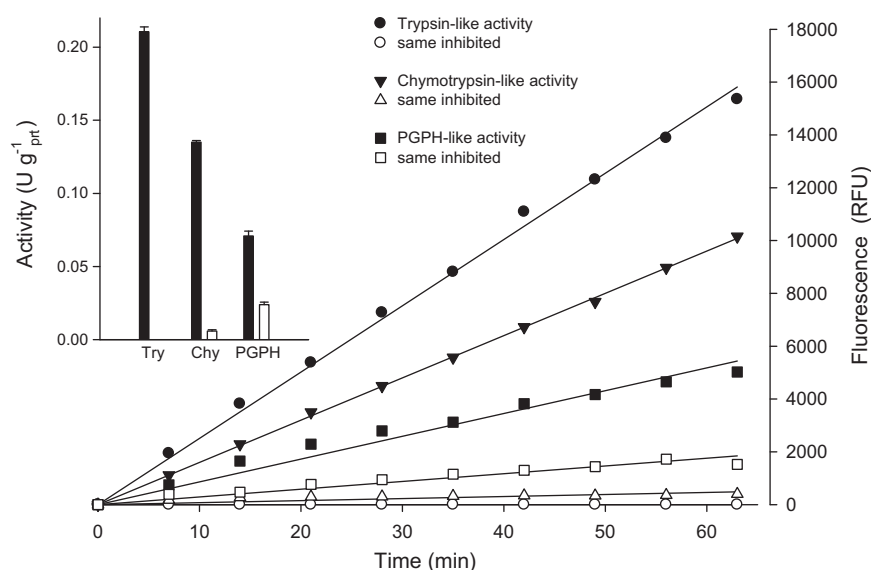


Fig. 2. Increase of fluorescence over time due to the hydrolysis of specific proteasomal substrates. Parallel assays were inhibited with epoxomicin. Averaged activities of the three proteasomal enzymes are presented in the inset as bar charts. The black symbols and bars represent the uninhibited activities, and the white symbols and bars represent the inhibited activities (means \pm standard errors, $n = 3$).

advantage of using NanoDrop fluorometry in enzymology is the reduction of sample amount, substrates, and other reagents such as cofactors and inhibitors. The method can be easily adopted for other samples and substrates. The reduced sample volume allows measurements on small organisms such as invertebrate larvae or parts thereof, tissue samples obtained by biopsy, and small amounts of cells from primary cultures or cell lines. The small scale reduces the amount of reagents required for a single measurement and simultaneously allows analyzing a larger number of individual samples, thereby strengthening the statistical validity of any study.

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