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Low-cost laser-induced fluorescence detector for micellar capillary zone electrophoresis

Detection at the zeptomol level of tetramethylrhodamine thiocarbamyl amino acid derivatives

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ABSTRACT

Tetramethylrhodamine thiocarbamyl (TRTC) amino acid derivatives are determined by micellar capillary zone electrophoresis with laser-induced fluorescence detection. A post-column fluorescence cell, based on a sheath-flow cuvette, is used as the detection chamber. A low-cost and low-power helium–neon laser, operating in the green at 543.5 nm, is used to excite the TRTC-amino acids. The relatively long excitation wavelength, the low-light scatter of the sheath-flow cuvette and the low excitation power result in a very low background signal, which is dominated at room temperature by detector dark current. To minimize the dark current, a cooled photomultiplier tube was used for photodetection. Detection limits (3σ) are $1 \cdot 10^{-21}$ mol (600 molecules) injected onto the capillary.

INTRODUCTION

Isothiocyanate derivatives of amino acids are of interest in Edman degradation sequence determination of proteins. The classic Edman reagent, phenyl isothiocyanate (PITC), is universally used in commercial protein sequencers [1]. The products of the sequencing reaction, phenyl thiohydantoin (PTH) derivatives of amino acids, have been separated by capillary electrophoresis and detected by ultraviolet absorbance [2]. Detection limits are about 100 fmol of the PTH derivatives with UV transmission detection. Other isothiocyanates may be used in the sequencing reaction. For example, dimethylaminoazobenzene isothiocyanate is used to produce the non-fluorescent dimethylaminoazobenzene thiohydantoin (DABTH) derivative that absorbs in the blue portion of the spectrum, with detection limits of *ca.* 100 fmol with liquid chromatographic separation and transmission detection. Sub-femtomole detection limits are produced for the PTH and DABTH derivatives with capillary electrophoretic separation and thermo-optical absorbance detection [3,4].

Fluorescent isothiocyanates can produce excellent detection limits for amino acids, roughly eight orders of magnitude superior to classic absorbance detection limits. This research group has described high-sensitivity detection of fluorescein isothiocyanate (FITC) derivatives of amino acid [3,5,6]. Fluorescein has strong absorbance at the argon ion laser emission wavelength of 488 nm, has high fluorescent quantum efficiency (*ca.* 0.5), but suffers from mediocre photobleaching properties [7]. The best detection limits reported by this laboratory for fluorescein thiohydantoin and thiocarbamyl derivatives of amino acids range from 1 to 2 zepto (10^{-21}) mol of derivative injected onto the capillary. Recently,

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other laboratories have reported detection limits of 10–20 zeptomol for fluorescein thiocarbamyl amino acid derivatives [8,9].

Tetramethylrhodamine isothiocyanate (TRITC) is an interesting modified Edman degradation reagent. The molecule has similar structure to FITC and should have similar reaction characteristics. TRITC absorbs strongly in the green portion of the spectrum and emits in the red. TRITC has been used to label immunological reagents for histological applications [10]; the red emission of TRITC contrasts nicely with the green emission of FITC in double-labeling experiments. The same chromophore is used by Applied Biosystems as their TAMRA-labeled primer for DNA sequencing.

TRITC offers useful spectroscopic properties for ultra-sensitive detection. The maximum absorbance is greater than 100 000 l mol^{-1} cm^{-1} at 540 nm while the emission maximum is 567 nm [10]. Soper *et al.* [11] have reported a fluorescence quantum yield of 15% and a photodestruction rate of $5 \cdot 10^{-6}$ in aqueous solutions. From a practical standpoint, the most important property of the dye is that is conveniently excited by the recently commercialized green helium–neon laser, which produces a 0.75-mW beam at 543.5 nm. The longer-wavelength excitation, compared with 488 nm excitation of fluorescein, produces a decrease in background signal due to the ν^4 property of light scatter. This green helium–neon laser is similar in construction to the conventional red helium–neon laser and should have similar lifetime, cost and power.

In this paper, we describe the use of TRITC to label amino acids for separation by capillary zone electrophoresis. While this research group has produced zeptomole detection limits for TAMRA-labeled DNA fragments separated by capillary gel electrophoresis [12,13], we know of no applications of the fluorophore for determination of small molecules by electrophoresis. The excitation of TRITC-labeled analyte with the green helium–neon laser results in a low-cost fluorescence detector with detection limits of 1 zeptomol.

EXPERIMENTAL

Separation was carried out with a 92 cm × 50 μm I.D. fused-silica capillary. The separation buffer was 5 m*M* pH 9.0 boric acid buffer with 10 m*M* sodium dodecyl sulphate. Electrophoresis was driven at 30 kV; the high-voltage (injection) end of the capillary was placed in a safety interlock-equipped Plexiglass box. Sample was injected electrokinetically at 1 kV for 10 s. The detector was locally constructed and is described below.

The beam from a 0.75-mW, 543.5-nm-wavelength, helium–neon laser (Melles Griot, Canada) was focused with a 4× microscope objective about 200 μm below the exit of the capillary in a post-column fluorescence flow chamber. The fluorescence detector is based on a sheath-flow cuvette and is similar to that used in FITC detection. The locally assembled cuvette has a 200-μm^2 flow chamber (NSG-Precision Cells, Farmingdale, NY, USA) and 2-mm-thick windows. Fluorescence is collected at right angles with an 18×, 0.45 numerical aperture objective (Melles Griot) and imaged onto a 0.8-mm-diameter pinhole. A single interference filter (590 nm center, 40 nm band pass, Model 590DF35, Omega Optical, Vermont, NE, USA) blocks scattered laser light while passing much of the fluorescence. Fluorescence was detected with a Hamamatsu (CA, USA) R1477 photomultiplier tube, operated at 1000 V and cooled to −25°C with a Products for Research (MA, USA) photomultiplier tube cooler. The photomultiplier tube output was passed through a 0.1-s resistor-capacitor (RC) low-pass filter and displayed on a strip-chart recorder.

Stock amino acid solutions were prepared in 0.2 *M* pH 8.6 boric acid buffer. Tetramethylrhodamine thiocarbamyl (TRTC) amino acid derivatives were produced by reacting 800 μl of $7 \cdot 10^{-5}$ *M* TRITC (Molecular Probes, Eugene, OR, USA) with 500 μl of 1–3 m*M* amino acid solution which was allowed to react at room temperature for 20 h in the dark. The TRTC-amino acids were diluted to the desired concentration with the separation buffer.

RESULTS AND DISCUSSION

The reaction of TRITC with amino acids is rather sluggish at room temperature. Samples of TRTC-glycine were allowed to react over a 10-h period. Small samples were removed from the reaction mixture and analyzed electrophoretically. The average of two or three determinations of the TRTC-amino acid peak height is plotted in Fig. 1; the data are connected by lines as a visual aid. The

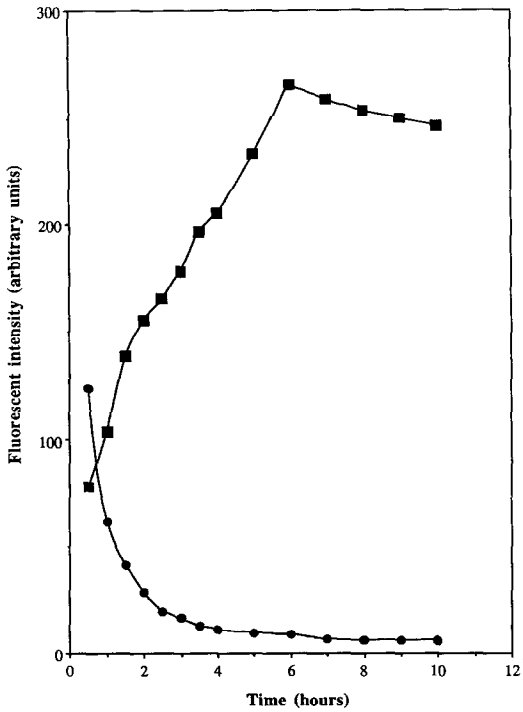

Fig. 1. Reaction efficiency of tetramethylrhodamine isothiocyanate with glycine. ■ = TRTC-glycine; ● = TRITC.

squares correspond to the production of TRTC-glycine while the circles correspond to unreacted TRITC. In this reaction, the amino acid was present in nearly eighteen-fold excess, so that consumption of TRITC and production of TRTC-amino acid are expected to follow first-order kinetics with the same rate constant. Instead, TRITC disappears more rapidly than TRTC-glycine is produced. It appears that TRITC undergoes both reaction with the amino acid and decomposition. Similar behavior has been observed in this laboratory for FITC reaction with amino acids. The isothiocyanates are not particularly stable in protic solvents, presumably undergoing hydrolysis to generate non-fluorescent products. At room temperature, TRITC disappears after about 6–8 h. Analyte concentrations are calculated based on TRITC as the limiting reagent and the assumption that the reaction goes to completion. As a result, the instrumental detection limits quoted below must be interpreted as being upper bounds to the actual detection limit.

Fig. 2 presents the electropherogram of twenty

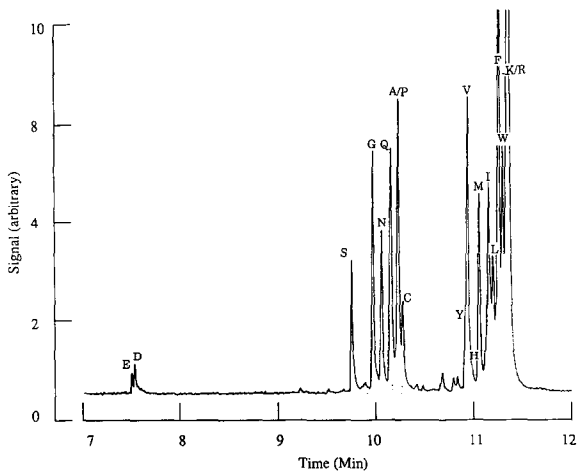

Fig. 2. Electropherogram of twenty TRTC-amino acids. The symbols for each amino acid correspond to the standard one-letter abbreviation.

amino acids. As is typical for zone electrophoretic separation of other thiocarbamyl derivatives of amino acids, separation is not complete; alanine and proline co-elute as do lysine, arginine and TRITC. However, the separation of the remaining amino acids is good and the total separation time is 12 min. On the other hand, the theoretical plate count for the separation is on the order of $2 \cdot 10^6$ plates. This separation demonstrates that high plate counts do not automatically translate into high resolution. The large size of the derivatizing reagent dwarfs small differences in the size of the amino acids. The size-to-charge ratio of the amino acid derivatives is quite similar and baseline separation is not achieved.

Detection limits are calculated by the method of Knoll [14]; the baseline is inspected over a time period given by 50 times the peak width. The maximum deviation from the average of the baseline is used to estimate the 3σ detection limit. This technique, based on the Tchebycheff inequality [14], relies on robust statistics. Very weak assumptions are made on the underlying noise distribution in the data. More importantly, correlation introduced between adjacent datum due to filtering does not artificially underestimate the standard deviation of the baseline. Relatively precise estimates of the detection limit are produced by inspecting baseline over a large number of samples. The 3σ detection limit for TRTC-glycine was $1 \cdot 10^{-12}$ M labeled amino acid

injected onto the capillary. Injection volume was estimated to be 1 nl for this amino acid, so that detection limits correspond to 1 zeptomol of amino acid injected onto the capillary. A 1-zeptomol amount of analyte corresponds to 600 analyte molecules. The detection volume, defined by the intersection of the laser beam and sample stream, was estimated to be 10 pl. There are 5 analyte molecules present on average in the detection volume at the detection limit.

Isothiocyanate derivatives are of interest for protein sequencing. However, given the sluggish nature of the isothiocyanate reaction, other derivatizing reagents are of interest for general amino acid analysis. The most interesting reagents are those that absorb strongly at the excitation wavelength of readily available lasers [15]. Derivatizing reagents that have been coupled with laser-induced fluorescence detection include *o*-phthalaldehyde excited by an argon ion laser operating in the ultraviolet region at 351 and 363 nm [16], Dansyl excited by a helium–cadmium laser at 325 nm [17], naphthalenedialdehyde (NDA) excited by the 457.9-nm line of an argon ion laser [18], fluorescamine-labeled amino acids excited at 325 nm with a mercury lamp [19] and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde excited at 442 nm with a helium–cadmium laser [20]. It will be interesting to see if zeptomole amino acid analysis is possible with these derivatives.

CONCLUSIONS

TRITC may be used to label amino acids, producing the thiocarbamyl derivatives. These derivatives may be separated by capillary electrophoresis and detected with high sensitivity by a low-cost laser-induced fluorescence system. Detection limits are 1 zeptomole of TRTC-glycine injected onto the capillary.

Tetramethylrhodamine isothiocyanate reacts relatively slowly with amino acids; the reaction rate is far too sluggish for routine use in protein sequencing. The classic Edman reagent, PITC, will react in less than 30 min. An equal-concentration mixture of PITC and TRITC can be used in a double-coupling sequencing reaction. The reaction with both reagents will yield a large excess of the PTH and a small amount of tetramethylrhodamine thiohydantoin after each step in the Edman cycle. The PITC is not detected but instead used to scavenge any unreacted *n*-terminal amino acids. The tetramethylrhodamine-labeled amino acids can be detected with exquisite sensitivity. Even if the tetramethylrhodamine reaction efficiency is 0.1% of the reaction efficiency of PITC, proteins may be sequenced with attomol sensitivity.

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