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RAPID ASSAY FOR TRYPTOPHANASE USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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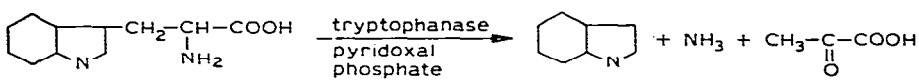
SUMMARY

A rapid and sensitive high-performance liquid-chromatographic assay for tryptophanase based upon the fluorometric measurement of the enzymatically liberated indole was developed. The total incubation time is 20 min, and the reversed-phase separation is fast (elution time of indole is 8 min) and reproducible. The sensitivity of the method is in the nanomole range. This method was tested in the assay of tryptophanase activity in *E. coli*, giving an average activity of 6589.6 U/g of cells. Because of its speed, high sensitivity and minimal sample preparation, this method circumvents several problems commonly encountered in standard spectrophotometric methods of analysis.

INTRODUCTION

The determination of tryptophanase activity in different microorganisms is usually based upon the determination of indole enzymatically produced according to the following reaction:



The indole content is then analyzed colorimetrically following its reaction with aldehydes in strong mineral acids[1–3] or the formation of nitrosoindole derivative[4]. Since the substrate, tryptophan, and the reaction product, indole, have close structural similarity, interferences are often encountered in colorimetric analyses unless the indole is extracted in organic solvents[1–3]. In addition, most commonly used colorimetric methods for the determination of indole in tryptophanase assays are usually time-consuming, tedious, or not adequately reproducible and quantitative. Therefore, they do not lend themselves to rapid assaying of tryptophanase activity in biological systems.

High-performance liquid chromatography (HPLC) has tremendous potential for enzyme assays[5] because of its great separating power, speed of analysis and reliable quantitation. In addition, since fluorometric detection offers a highly sensitive

and selective tool for detecting minute amounts of naturally-fluorescing or fluorescent-labeled compounds, it is ideally suited for the detection of indoles[6].

Therefore, the use of an isocratic elution mode of reversed-phase HPLC was investigated in the determination of tryptophanase activity in *E. coli*.

EXPERIMENTAL

A Model 6000A solvent delivery system, Model 660 solvent programmer and Model U6K universal injector, all from Waters Assoc. (Milford, Mass., U.S.A.). were used in all determinations. A FS 970 fluorescence monitor with a deuterium lamp, SFA 339 wavelength drive and MM 700 memory module (Kratos, Schoeffel Instrument Division, Westwood, N.J., U.S.A.) were used for detection and identification of the column effluents by means of their stopped-flow excitation spectra.

A prepacked, stainless-steel column, μBondapak C_{18} (10 μm particle size) was obtained from Waters Assoc.

For the preparation of tryptophanase extracts from *E. coli*, a Fisher sonic dismembrator, Model 300 with an intermediate probe tip (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was used.

Peak areas were electronically integrated using a Hewlett-Packard Model 3380-A integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Reagents

All reagents used were of the highest purity (ACS certified grade). Reference compounds, including lyophilized *E. coli* cells enriched in tryptophanase, were purchased from Sigma (St. Louis, Mo., U.S.A.), methanol, distilled in glass, from Burdic & Jackson Labs. (Muskegon, Mich., U.S.A.), and potassium dihydrogen phosphate from Mallincrodt (St. Louis, Mo., U.S.A.).

Solutions of reference compounds were prepared in distilled, deionized water and kept frozen until use.

Chromatographic conditions

Samples were eluted isocratically using anhydrous methanol–water (1:1, v/v) which was always degassed before use. The flow-rate was 1.0 ml/min and the temperature was ambient in all cases.

Chromatographic peaks were monitored flourometrically using an excitation wavelength of 285 nm and an emission cut-off filter of 320 nm.

Preparation of samples

Approximately 0.5 g *E. coli* cells were suspended in 5 ml of 0.2 *M* potassium dihydrogen phosphate, buffered to pH 7.0, and the suspension was sonicated for 5 min to extract the tryptophanase. Portions of the extract were first incubated at 37° for 10 min with 0.2 ml of pyridoxal phosphate (100 μg/ml) and then with an equal volume of 0.5 *M* tryptophan for 10, 20 and 40 min. The standard procedure is summarized in Scheme I.

For the deproteination of samples, several methods were tried: heat, addition of 1 ml of 6% by volume trichloroacetic acid (TCA), and addition of 1 ml of saturated ammonium sulfate solution to each millilitre of extract.

(1) To 0.5 g lyophilized *E. coli* cells add 5 ml of 0.2 *M* KH_2PO_4 and buffer to pH 7.0
(2) Sonicate for 5 min
(3) To 0.2 ml of extract, add 0.2 ml of 100 μg/ml pyridoxal phosphate
(4) Incubate for 10 min at 37°
(5) Add 0.2 ml of 0.5 *M* tryptophan
(6) Incubate for 10, 20 or 40 min at 37°
(7) Terminate the reaction by addition of 0.2 ml of TCA
(8) Neutralize the acid extract with solid tris(hydroxymethyl)aminomethane
(9) Centrifuge for 10 min at 630 *g*
(10) Analyze supernatant by HPLC

Scheme I. Protocol conditions for tryptophanase assay by reversed-phase HPLC.

Extracts were then vortexed at a moderate speed for 30 sec and centrifuged at 630 *g* for 5 min. The supernatant was filtered through a Millipore membrane filter, type HA pore size 0.22 μm (Millipore, Bedford, Mass., U.S.A.) and immediately chromatographed. In the procedure using TCA precipitation, extracts were neutralized using solid tris(hydroxymethyl)aminoethane.

Identification of chromatographic peaks

Several identification methods were used to characterize chromatographic peaks. Initial peak identification was based on retention times. Extracts were then co-chromatographed with the reference compounds and quantitative increase in the area of suspected peak was taken as further evidence of its identity. Since it is possible that different compounds may elute with identical retention times, the stopped-flow excitation spectra were also obtained. In order to obtain the excitation spectra, first a blank gradient was run and the flow stopped at the point where the compound of interest elutes. The background excitation spectrum, which arises from the change in optical properties of the solvent and the photomultiplier response with wavelength, is stored in the memory unit and automatically subtracted from the scan of the sample peak to give a corrected spectrum. The described methods were used in combination to determine the identity and the purity of the compounds under study.

DISCUSSION

For the determination of indole, enzymatically produced from tryptophan, an isocratic elution mode of reversed-phase HPLC was used. The separation of a synthetic mixture of two reference compounds detected fluorometrically is shown in Fig. 1.

Prior to the chromatographic assay of tryptophanase activity in *E. coli*, the extracts were deproteinated and the sample filtered to avoid contamination of the column. Several standard methods of protein removal were tested, such as the use of TCA, heat and ammonium sulfate precipitation.

Chromatograms of the incubated *E. coli* extract analyzed immediately after deproteination by heat ($t = 0$) and 45 min and 1.67 h, respectively, are shown in Fig. 2. As can be seen from the illustration, 1–2 min heating of the sample in boiling water did not effectively deactivate the enzyme, so that indole continued to form. Increased heating time (5–10 min) proved effective in deactivation of the

enzyme. However, because of the high protein content of the extract, prolonged heating resulted in formation of a gel which made filtration of the sample difficult.

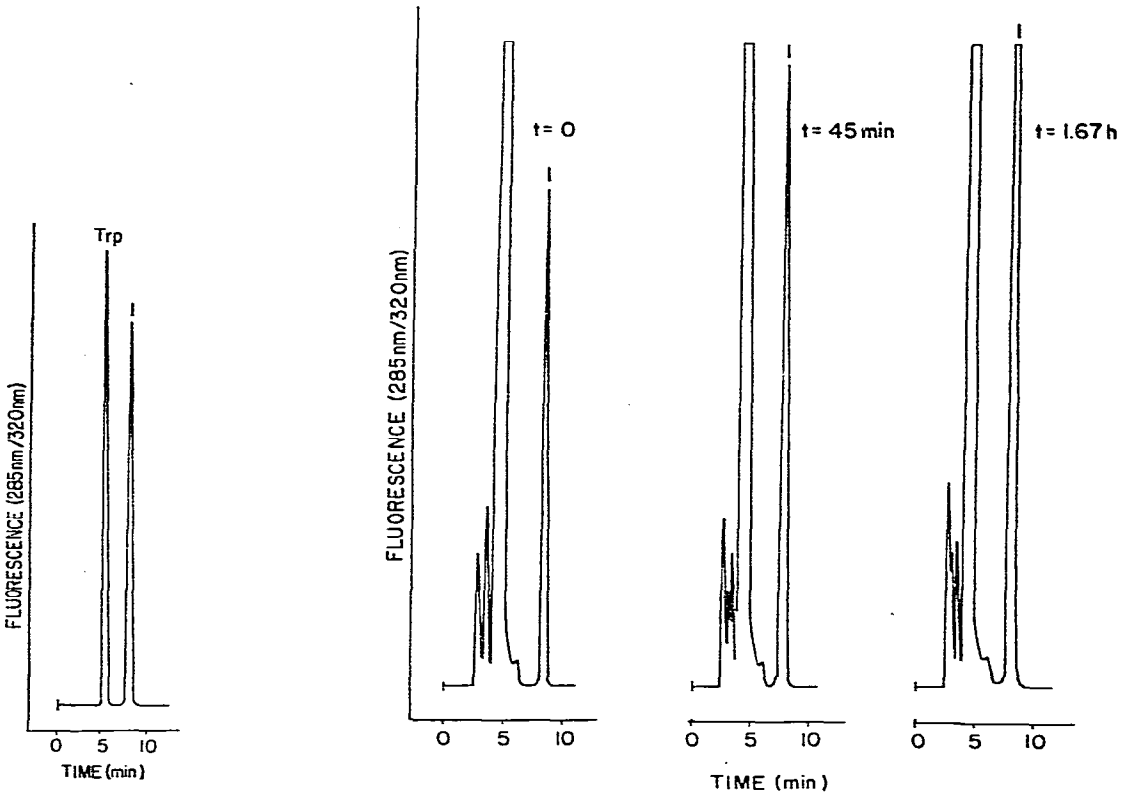


Fig. 1. Separation of a synthetic mixture of tryptophan (Trp) and indole (I). Chromatographic conditions: column, μBondapak C_{18}; eluent, anhydrous methanol–water (1:1, v/v); flow-rate, 1.0 ml/min; temperature, ambient; detection, fluorescence, 285 nm excitation, 320 nm emission cut-off filter.

Fig. 2. Chromatograms of the *E. coli* extract incubated with tryptophan for 10 min and deproteinated by heat. Incubation conditions given in Scheme I. The sample was chromatographed immediately after deproteination ($t = 0$) and after 45 min and 1.67 h, respectively. Chromatographic conditions same as in Fig. 1. Volume injected, 6.6 μl; attenuation, 0.5 μA.

Precipitation of protein with a saturated solution of ammonium sulfate was also tried (Fig. 3). As can be seen from the illustration, the incubated extract deproteinated by this method is stable for more than 2 h. However, this method results in incomplete protein removal which can cause deterioration of the column. In addition, the reproducibility was also found to be poor, and therefore this method was not adopted for the protocol procedure.

TCA proved to be the most efficient method for the removal of protein. However, it should be noted that because of the gradual break-down of indole in highly acidic solutions, the extracts had to be neutralized with solid tris(hydroxymethyl)aminomethane. Repeated injections of the incubated acidic extract shown

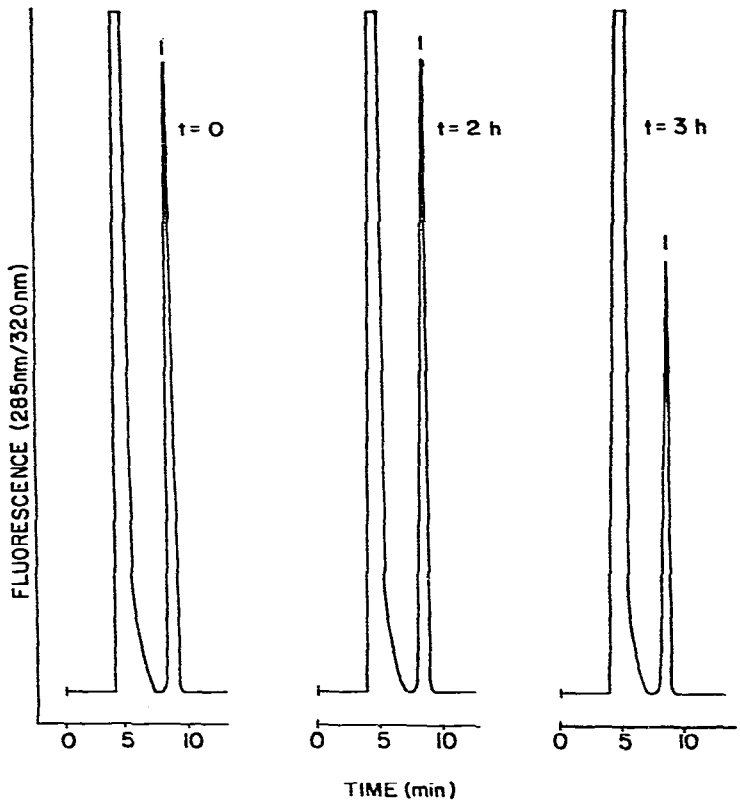


Fig. 3. Chromatograms of the *E. coli* extract incubated with tryptophan for 10 min and deproteinated with ammonium sulfate. Incubation conditions given in Scheme I. The sample was chromatographed immediately after deproteination ($t = 0$) and after 2 and 3 h, respectively. Chromatographic conditions same as in Fig. 1. Volume injected, 8.4 μl; attenuation, 0.5 μA.

in Fig. 4 illustrate a rapid change in the indole concentration over a short period of time. The break-down product elutes immediately before the indole peak.

Freezing of the incubated acidic extracts proved to increase the rate of indole decomposition, probably due to the formation of micro-regions of elevated TCA concentration in the structure of ice. However, neutralization of the TCA extracts gave reproducible results. The solutions were stable for at least 3 h and therefore this method of protein removal was adopted and used throughout the study.

Once the proper deproteination method had been selected, it was necessary to determine whether the *E. coli* cells contained any naturally-occurring indole. In order to ensure that the indole found in the sonicated extracts, which were not incubated with tryptophan did not result from the acid break-down of the cell constituents, a sample of sonicated *E. coli* cells was only filtered and no TCA was added to it. As can be seen from Fig. 5, the indole peak in the extract with no TCA added (a) and the extract deproteinated with TCA (b) were identical which indicates that the indole found is endogenous.

After optimization of the reaction conditions, the standard protocol procedure was adopted (Scheme I). Prior to the quantitation of indole enzymatically liberated

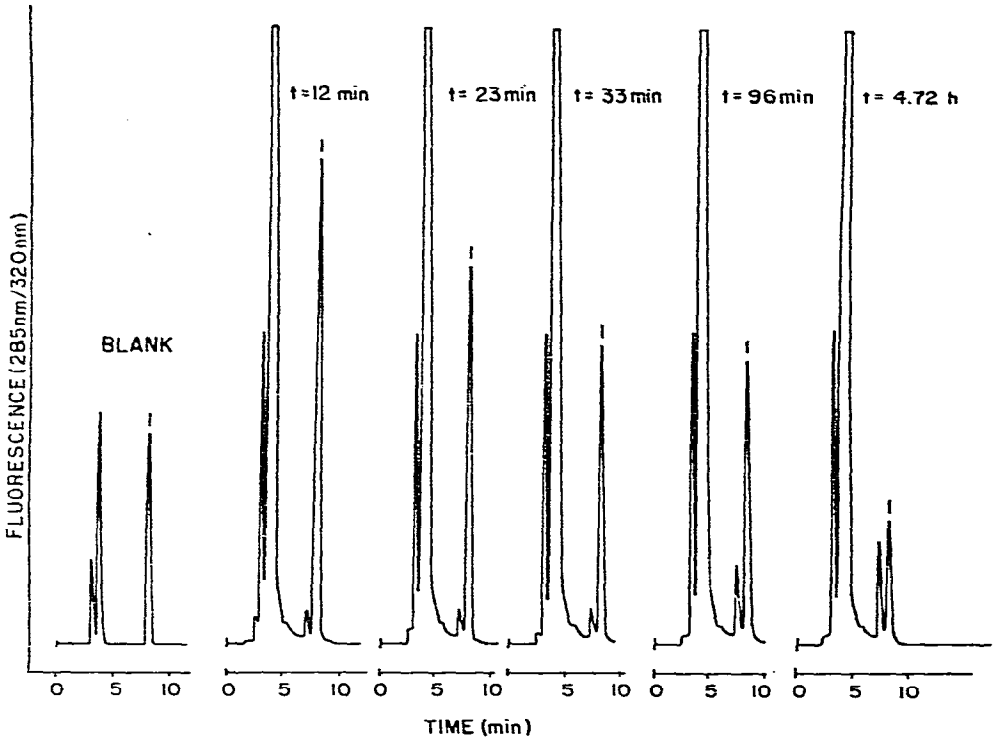


Fig. 4. Time study of indole decomposition in acidic solution. Sample: *E. coli* extract incubated with tryptophan for 10 min. Incubation conditions same as in Scheme I except for addition of solid tris(hydroxymethyl)aminomethane. Chromatographic conditions same as in Fig. 1.

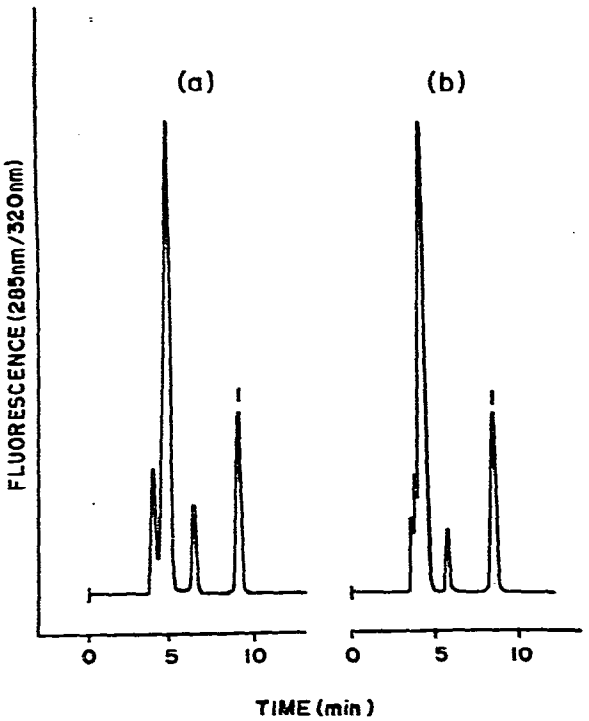


Fig. 5. (a) Chromatogram of the *E. coli* extract deproteinated with TCA. (b) Chromatogram of the *E. coli* extract filtered through a 0.22-μm Millipore filter. No TCA added. Chromatographic conditions as in Fig. 1.

from *E. coli*, a plot of the instrument response (peak height) as a function of indole concentration was obtained. The detector response was found to be linear with concentration over the entire working range. In addition, the lower detection limit was also determined and it was found to be approximately 10 nmoles.

In order to ensure favorable kinetics, *E. coli* samples were incubated with a large excess of tryptophan. The reaction was stopped by addition of cold, freshly-prepared TCA and the extracts were then neutralized with solid tris(hydroxymethyl)aminomethane. The results of the 10, 20 and 40 min incubations are shown in Fig. 6.

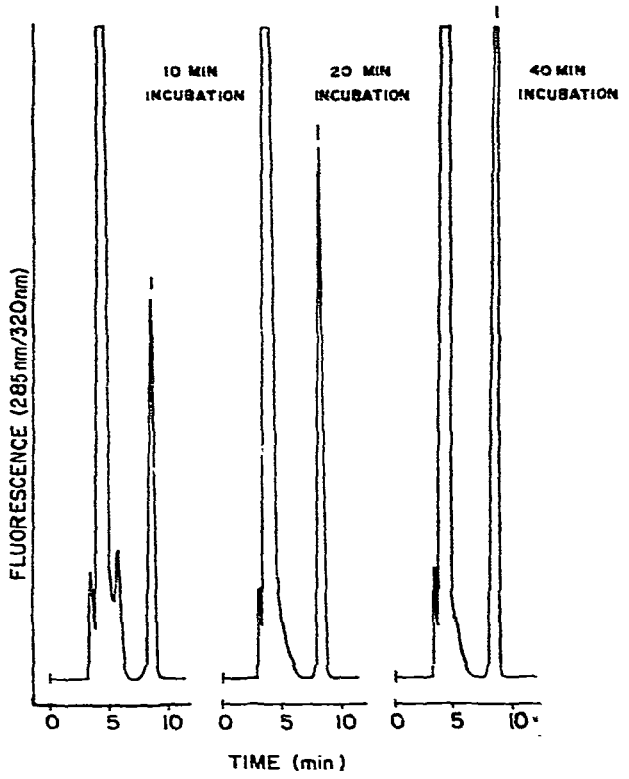


Fig. 6. Chromatograms of the *E. coli* extracts incubated with tryptophan for 10, 20 and 40 min, respectively. Samples were deproteinated with TCA, and the extracts neutralized with solid tris-(hydroxymethyl)aminomethane. Chromatographic conditions same as in Fig. 1. Volume injected, 5 μl; attenuation, 0.5 μA.

The activity of the enzyme was calculated from the concentration of the enzymatically-produced indole, using the following formula:

$$\text{Activity (U/g of } E.\ coli) = \frac{\text{area} \cdot \text{total vol.}}{\text{response factor} \cdot (\text{vol. inj.} \cdot 10^{-3}) \cdot \text{reaction time} \cdot \text{wt. of sample}}$$

$$\text{Area} = (\text{std. area})\,(\text{dilution factor}) - \text{area in extract}$$

$$\text{Response factor (area/}\mu\text{mole)} = \frac{\text{std. area}}{(\text{vol. inj.} \cdot 10^{-3}) \cdot \text{std. conc. } mM}$$

The activities of the enzyme calculated for the incubation times of 10, 20 and 40 min were found to be 6589.8, 6470.4, and 6708.5, respectively. Therefore, the average tryptophanase activity was 6589.6, and the standard deviation of the three measurements was 1.81%.

CONCLUSIONS

The described reversed-phase HPLC determination of tryptophanase activity offers several advantages over currently available assays. Sample preparation is minimal and it involves only precipitation of protein prior to chromatography. The analysis time, after incubation, is short (8 min) and the separation is free from interferences. The enzymatically-liberated indole is measured fluorometrically which makes the detection highly sensitive and selective. Simple online identification method assures unambiguous identity of the chromatographic peaks.

This method is illustrated with the assay of tryptophanase in *E. coli*, but it can be applied to other biological samples. In addition, it can also be adopted for identification of tryptophan in biological samples. If the sample is incubated with tryptophanase, tryptophan will be enzymatically converted to indole, which elutes with a different retention time. This method, known as the enzymatic peak-shift, exploits the specificity of enzymatic reactions and it has proven to be a simple and elegant method of peak identification[7].

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