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A rapid and specific colorimetric method for free tryptophan quantification

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ABSTRACT

Tryptophan is one of the eight essential amino acids and plays an important role in many biological processes. For its interaction with human health, environment and relevant commercial interest in biotechnology-based production, rapid and specific quantification method for this molecule accessible to common laboratories is badly needed. We herein reported a simple colorimetric method for free tryptophan quantification with 96-well-plate-level throughput. Our protocol firstly converted tryptophan to indole enzymatically by purified tryptophanases and then used reactivity of indole with hydroxylamine to form pink product with absorption peak at 530 nm, enabling the quantification of tryptophan with simple spectrometry in just two hours. We presented that this method exhibited a linear detection range from 100 μM to 600 μM (R^2 = 0.9969) with no detection towards other naturally occurring tryptophan analogs or tryptophan residues in proteins. It was very robust in complicated biological samples, as demonstrated by quantifying the titer of 36 mutated tryptophan-producing strains with Pearson correlation coefficient of 0.93 in contrast to that measured by high performance liquid chromatography (HPLC). Our method should be potent for routine free tryptophan quantification in a high-throughput manner, facilitating studies in medicine, microbiology, food chemistry, metabolic engineering, etc.

1. Introduction

Tryptophan is an important building brick of proteins, to a large extent determining protein properties such as activity [1], hydrophobicity [2], structure [3], etc. Tryptophan is also an active player in many biological processes, whose metabolism significantly impacts human health [4]. The rapid and specific quantification of tryptophan is thus of great interest for either its biochemical study or microbe derived bioproduction [5]. However, due to the naturally occurring coexistence with other amino acids, the measurement of tryptophan suffers from serious interference and usually a separation step is necessary such as HPLC [6], which often resulted in poor throughput, causing problem when rapid analysis of a big amount of samples was needed. Over the past several decades, intensive efforts have been devoted to seeking direct approaches (separation free) to quantify tryptophan in a mixture of amino acids with higher throughput. These efforts have thus far involved multiple disciplines such as molecular recognition [7,8] and electrochemical assays [9]. However, these methods require expensive instruments or complicated synthetic chemistry, which is not accessible to common laboratories without the relevant expertise.

In contrast, colorimetric methods based on simple reagents can overcome the aforementioned disadvantages, providing a simple approach for routine tryptophan measurement. Ninhydrin derived chemistry is the most commonly used method for tryptophan detection. However, interference from other amino acids, especially cysteine and tyrosine [10,11] limited its utilization in many scenarios. A simpler colorimetric method based on the reaction of tryptophan with formic acid in the presence of hydrochloric acid was developed [12], which was further optimized by the addition of DMSO, enabled the reaction to complete at room temperature within minutes [13]. This method exhibited great specificity towards tryptophan over other amino acids. However, it failed to quantify free tryptophan due to the co-reaction with tryptophan residues of proteins in the sample, impairing its utilization in many biological samples where proteins might be a main contamination source.

To improve the specificity of detection towards free tryptophan, enzyme reaction might be an alternative. In fact, there are many enzyme-based assays for different kinds of chemicals, such as allopurinol, endotoxin, glycerol, glucose, methanol, nitrate, nitrite and so on [14]. For example, for detection of nitrate and nitrite in biological samples, the most commonly used method is combination of nitrate reduction (reduced to nitrite) by nitrate reductase and spectrophoto-

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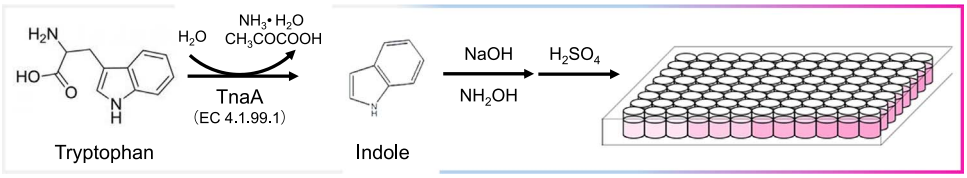

Fig. 1. Schematic diagram of high-throughput tryptophan assay (HTA).

metric assay of nitrite with Griess reagent [15,16]. Inspired by this, we presented a rapid but highly specific method, namely high-throughput tryptophan assay (HTA), for free tryptophan quantification in this work by combining tryptophan enzymolysis with indole-hydroxylamine chemistry. This indole-hydroxylamine chemistry was firstly described by Isenberg and Sundheim in 1958 [17] and later used to measure indole in complicated biological samples [18], in which hydroxylamine reacted with free indole in alkaline environment and exhibited pink color after the addition of sulfuric acid.

In this study, we cloned and purified tryptophanase TnaA from *Escherichia coli* to convert tryptophan to indole in vitro, thus enabling free tryptophan quantification by indole-hydroxylamine chemistry (Fig. 1). We optimized the protein purification process, especially the reagent to stabilize the protein, thus rendering the quantification more robust, with a detection limit to about 100 μM (signal:noise = 3:1) and linear range up to 600 μM. Our results showed that tryptophan residues in dipeptides, as well as other two aromatic amino acids exhibited minimal interference with the quantification. Diverse complicated biological samples were also tested to demonstrate the robustness of this method. Our method presented 96-well-plate-level quantification throughput within just two hours manipulation, thus should be potent as a rapid and specific approach for free (or total) tryptophan quantification enabling hundreds of samples processed simultaneously.

2. Experiments

2.1. Plasmid construction

DNA (PCR product) purification (D2500) and isolation of high quality plasmids (D6943) were performed using reagents from Omega Bio-Tek (U.S.). DNA amplification enzymes were derived from New England Biolabs (U.S.). Genomic DNA was purified using reagents from Tiangen Biotech (DP302) (Beijing, China). During plasmid construction, *E. coli* DH5α (BioMed, China) served as the host for molecular clone and was cultured in Luria-Bertani (LB) broth or on LB agar plates at 37 °C. Plasmids were constructed by Gibson assembly with customized recipe as described by the original paper [19]. We used PF/R_pET28a to linearize pET28a vector. The open reading frame (ORF) of *tnaA* was amplified by PF/R_tnaA_pET28a from the purified genomic DNA of *E. coli* DH1 (ATCC33849) with 6× His-tag inserted at N-terminal. Plasmid pET28a-*tnaA* was constructed by Gibson assembly with purified PCR product of linearized pET28a vector and *tnaA* ORF. This plasmid was found to have cellular toxicity in *E. coli* BL21(DE3) strain and abandoned later. Similarly, we constructed pTrc99a-*tnaA* by amplifying (his)6-*tnaA* from pET28a and inserting into linearized pTrc99a. 100 mg/L ampicillin was used to select and maintain pTrc99a-*tnaA* plasmid. All the oligonucleotides used in this work were purchased from Taihe Biotechnology Co., LTD (Beijing, China) and listed in Table 1.

2.2. Tryptophanase overexpression and purification

All chemicals used were purchased from Sigma Aldrich (U.S.). Plasmid pTrc99a-*tnaA* was transformed into *E. coli* BL21(DE3) strain (BioMed, China) for protein overexpression. Overnight culture in LB broth (100 mg/L ampicillin) at 37 °C from single colony was incubated into 100 mL fresh LB medium (100 mg/L ampicillin) in 500 mL shake flask and cultured at 30 °C. After optical density at 600 nm (measured by Amersham Bioscience spectrophotometer) reached around 0.8, isopropyl-β-D-thiogalactoside (IPTG) was added to final concentration of 0.1 mM. And the strain was further cultured at 30 °C for additional 24 h. The cell pellet was collected by centrifugation and washed with binding buffer (20 mM Na_2HPO_4, 500 mM NaCl, 30 mM imidazole, 10 mM mercaptoethanol or 1 mM TCEP, adjust pH to 7.4 by H_3PO_4) once. Then the harvested cells were suspended in binding buffer and disrupted by ultra-sonication (SCIENTZ BioTech Co. Ltd., Ningbo, China). The supernatant was obtained after centrifugation at 13,000× *g* for 30 min at 4 °C. Tryptophanase with 6× His-tag was purified using HisTrap HP columns (GE Healthcare, Chalfont St Giles, BUCKS, UK) with an AKTA prime chromatography apparatus (GE Healthcare, Uppsala, Sweden) by gradually increasing the fraction of washing buffer (20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, 10 mM mercaptoethanol or 1 mM TCEP, adjust pH to 7.4 by H_3PO_4) to 100% in 30 min. The purity of each fraction was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and highly purified fractions were combined. The protein concentration was determined by the Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). The purified enzymes was ultrafiltered to replace the washing buffer with KPbeta buffer (0.05 mM K_3PO_4/H_3PO_4, 100 μM pyridoxal 5′-phosphate, 10 mM mercaptoethanol or 1 mM TCEP, pH = 8.0) to > 99% (v:v). The composition of this buffer followed the protocol for tryptophanase activity characterization [20] with some adjustments in this work. Glycerol stock was prepared by mixing the enzyme solution with 50% glycerol (v:v) by 1:1 (v:v) to a final concentration of around 2 mg/mL and stored at −20 °C.

2.3. Hydroxylamine chemistry based tryptophan assay protocol

Indole, tryptophan, tyrosine, mercaptoethanol and TCEP were obtained from Sigma-Aldrich Co. Ltd. (U.S.). Alanine-tryptophan dipeptide was obtained from Tokyo Chemical Industry Co. Ltd. (Japan). Tryptophan-glycine dipeptide was obtained from Ark Pharm, Inc. (U.S.). All other chemicals were obtained from Beijing Chemical Factory (China). Stock solutions of indole (5 mM) were prepared in 70% ethanol (v/v). Stock solutions of tryptophan (5 mM), tyrosine (1 mM), phenylalanine (5 mM), Ala-Trp (5 mM), Trp-Gly (5 mM) were all prepared in ultrapure water. Standards were prepared in KP buffer (0.05 M K_3PO_4, 100 μM pyridoxal 5′-phosphate, adjust pH to 8.0 by H_3PO_4) by diluting the stock solutions to desired concentration. Before assay, we removed glycerol in TnaA stock with KPbeta buffer by ultrafiltration. The concentration of TnaA was measured and diluted to working concentration (20 μg/mL in final protocol) with KP buffer. Using 96-well plate (Corning Inc. U.S.), 100 μL 1:1 (v:v) mixture of standard or samples with KP buffer (used for control experiments) or TnaA enzyme working solution were incubated for 1 h (at 37 °C in the final protocol or 25 °C during optimization). Following incubation, 25 μL of 5.3 M NaOH and 50 μL of 0.3 M hydroxylamine (NH_2OH-HCl) were added and incubated at room temperature for 15 min, and then 125 μL of 2.7 M H_2SO_4 was added. After incubation at room temperature for 30 min, the absorbance of mixture at 530 nm was measured by Tecan 2500 Pro 96-well plate reader (Switzerland). It is important to note that optimum pH for TnaA was about 8.0 [21], to maximize the enzyme activity, the pH of

Table 1
All oligonucleotides used in this work.

Primer	Sequence	Usage
PF_tnaA_pTrc99a	aacaatttcacacaggaaacagaccATGCATCATCATCATCATCACATGGAAAAC	*tnaA* amplification for insertion into linearized pTrc99a
PR_tnaA_pTrc99a	tcttctctcatccgccaaaacagccTTAAACTTCTTTAAGTTTTGCGGTGAAGTGAC	*tnaA* amplification for insertion into linearized pTrc99a
PF_pTrc99a	ggctgttttggcggatgagagaag	pTrc99a linearization
PR_pTrc99a	ggtctgtttcctgtgtgaaattgttatc	pTrc99a linearization
PF_tnaA_pET28a	ATGCATCATCATCATCATCACATGGAAAACTTTAAACATCTCCCTGAACC	*tnaA* amplification for insertion into linearized pET28a
PR_tnaA_pET28a	GCAGCAGCCAACTCAGCTTCCTTTCTTAAACTTCTTTAAGTTTTGCGGTGAAGTGAC	*tnaA* amplification for insertion into linearized pET28a
PF_pET28a	GAAAGGAAGCTGAGTTGGCTGC	pET28a linearization
PR_pET28a	CCATGTGATGATGATGATGATGCATGCTGCTGCCCATGG	pET28a linearization

KP (or KPbeta) buffer should be adjusted according to the samples to be tested.

2.4. Tryptophan fermentation conditions

Single colonies of tryptophan producers were each inoculated into 1 mL LB broth in 48-well deep well blocks and cultured overnight at 37 °C. Then 20 µL seed culture was added into 2 mL fresh M9-YE medium (5 g/L glucose) [22] in 24-well deep well blocks and cultivated at 37 °C for 24 h. After that, 1 mL cell culture was centrifuged at 13,000 rpm for 10 min to get supernatant for further tryptophan analysis (by HPLC or HTA). The HPLC method for detecting tryptophan was as described by Fang et al. [5].

3. Results and discussion

3.1. Clone and purification of tryptophanase

We firstly sought to clone and purify tryptophanase TnaA (WP_001295247.1), a lyase capable of breaking tryptophan into ammonia, pyruvate and indole with pyridoxal 5′-phosphate as cofactor from *E. coli* DH1. We cloned *tnaA* into pTrc99a expression vector and used *E. coli* BL21(DE3) as host for protein overexpression. We followed standard protocol (see Experiments) for nickel column based purification and obtained both high yield (around 5 mg/100 mL culture) and purity for tryptophanase.

3.2. Establishment of the method

To accomplish the quantification of tryptophan with simple spectrometry, we used tryptophanases to hydrolyze tryptophan to indole for further detection with hydroxylamine. We firstly reproduced the results of Darkoh et al. [18] and found that hydroxylamine reacted and gave rise to pink color only with indole but not tryptophan in the described conditions (see Experiments) (Fig. 2a), suggesting that tryptophanase was required to establish the chemistry for free tryptophan quantification. However, when we tested the impact of essential components for tryptophanase activity (pyridoxal 5′-phosphate: cofactor; mercaptoethanol: reducing reagent responsible to protect the disulfide bond of tryptophanase) on hydroxylamine reaction, we found that 0.1 mM mercaptoethanol significantly interfered with the reaction. Specifically, in spite of appearance of pink color as expected, mercaptoethanol impaired the stability of the colored product, rendering the color to fade significantly after minutes. Hence, we replaced mercaptoethanol with another commonly used reducing reagent in protein chemistry, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and confirmed the stability of the colored product in the presence of 0.1 mM TCEP (Fig. 2b). TCEP was used for the following work described in this paper.

We consequently optimized the conditions of TnaA enzymatic reaction. We incubated tryptophan with purified tryptophanase in the optimized KPbeta buffer, containing essential ion and cofactor supporting enzyme activity (see Experiments), for 1 h and compared the enzyme activity at 25 °C or 37 °C (Fig. 3a) by further subjecting these mixtures to react with hydroxylamine. The result suggested that tryptophan was successfully quantified at either condition, but 37 °C incubation led to a prolonged linear range for quantification and thus chosen as the condition in our protocol. We then tested the influence of enzyme concentration. 40, 20 and 10 µg/mL tryptophanase (concentration referred to that in incubation with tryptophan) exhibited similar performance while 5 µg/mL led to significantly reduced sensitivity (Fig. 3b), which was in consistent with previously described specific activity data of tryptophanase [21]. Hence, 20 µg/mL enzyme was used to provide enough redundancy in our protocol. The standard curve of tryptophan assays at optimized conditions was shown in Fig. 3c, elucidating the linear detection range from 100 to 600 µM (from 0.020 to 0.122 g/L) with a linear regression coefficient of 0.9972.

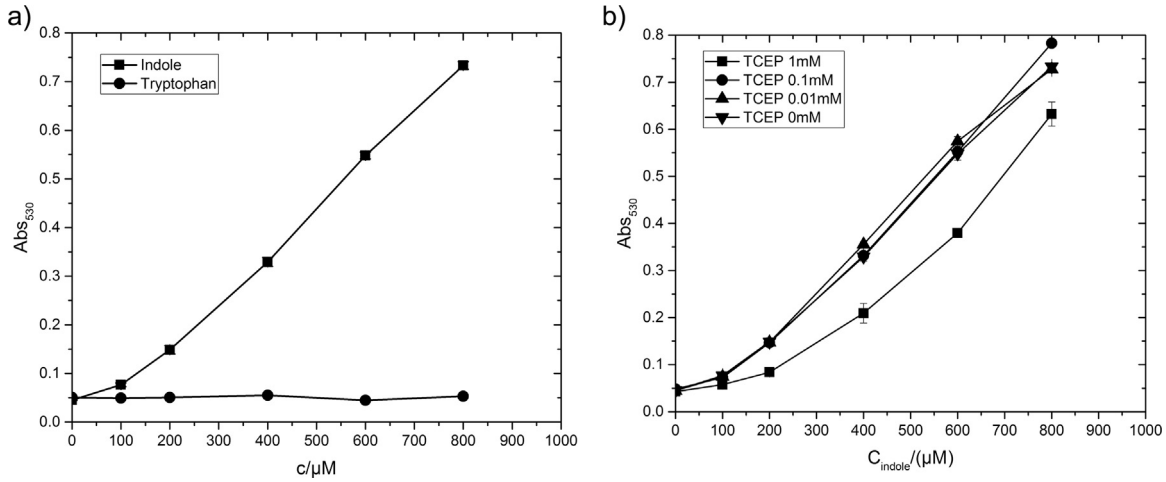

Fig. 2. Pre-test on feasibility of developing hydroxylamine-based indole assay into tryptophan assay. a) Specificity of hydroxylamine-based indole assay. Tryptophan cannot be detected by the hydroxylamine-based indole assay without the conversion via TnaA enzyme. b) Influence of TCEP concentration on hydroxylamine-based indole assay.

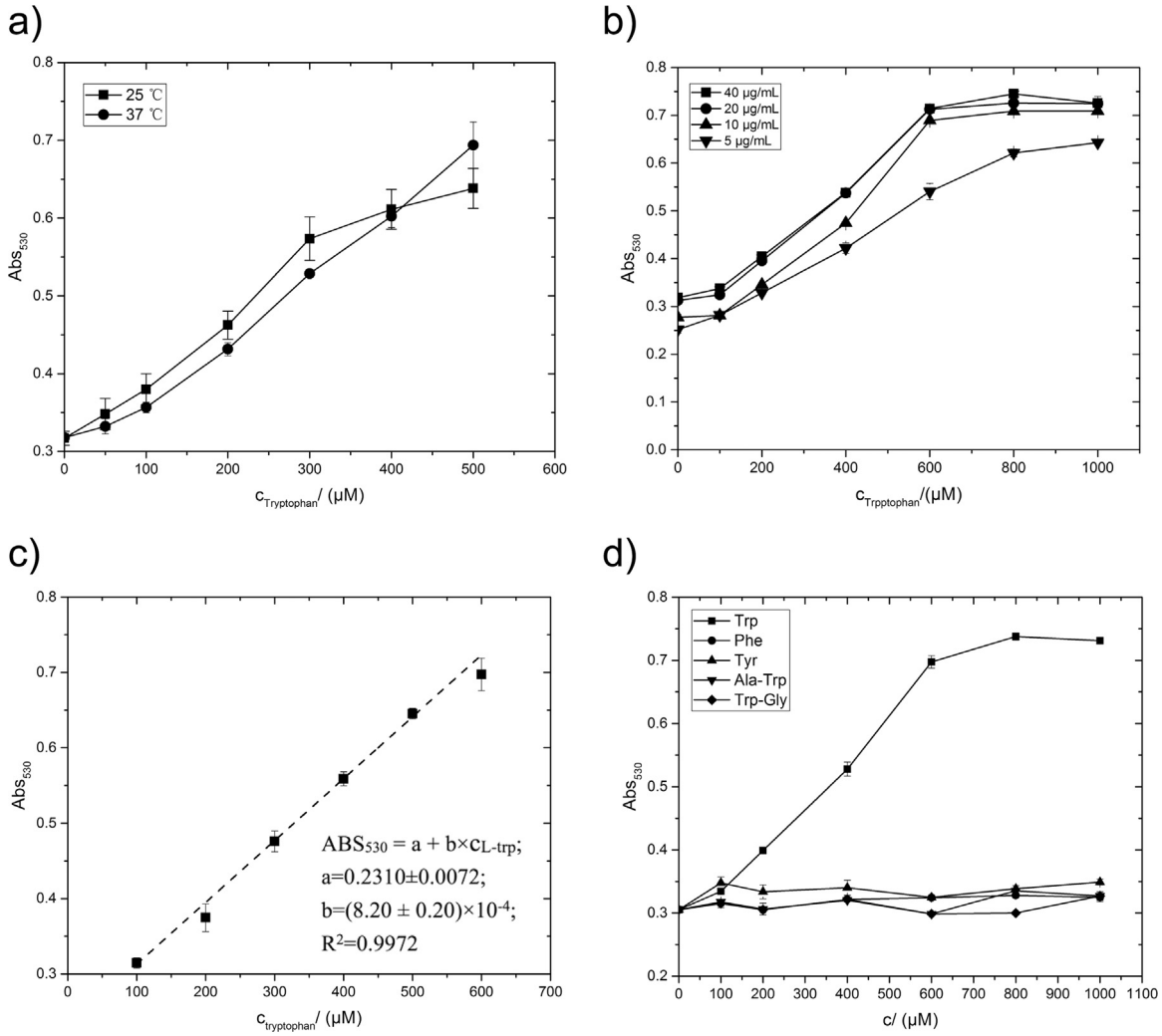

Fig. 3. Assay protocol optimization and substrate specificity analysis. a) Influence of enzymolysis temperature on tryptophan quantification based on hydroxylamine derived colored reaction. b) Influence of TnaA concentration on tryptophan assays. c) Standard curve of tryptophan assays. d) Substrate specificity of tryptophan assays.

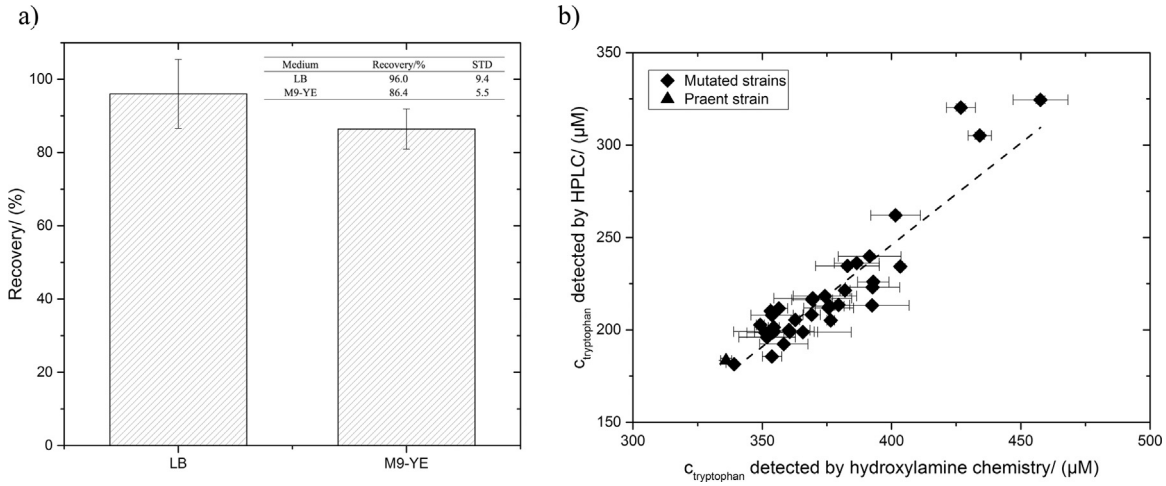

Fig. 4. Free tryptophan quantification in complicated biological samples. a) Recovery rate characterization of tryptophan in two mediums by adding 100 μM tryptophan. b) Application of the high-throughput tryptophan assay method to screen for tryptophan overproducers from a mutant library.

Finally, to verify the specificity of this method, two other aromatic amino acids, tyrosine and phenylalanine, and two kinds of dipeptides (alanine-tryptophan, Ala-Trp; tryptophan-glycine, Trp-Gly) as analogs of tryptophan residues in proteins, were characterized by the same protocol and no signal was detected (Fig. 3d). Together with the extensive study that hydroxylamine has poor reactivity with many indole derivatives [17,18], the high specificity of HTA was confirmed. And the results verified that hydroxylamine chemistry was highly specific for free tryptophan quantification. It should be noted that indole is a potential interfering chemical due to the chemistry of HTA

Table 2
Performance comparison of other established separation-free tryptophan analytical methods with this work.

$c_{L\text{-}trp}$/(μM)	Detection limit	Linear range	Specificity		Reaction condition
			Detection of aromatic amino acid	Detection of tryptophyl residues in peptides or proteins	
HCl-HCOOH [12]	100 μM	Not shown	–	+	50 °C 5 h
HCl-HCOOH-DMSO [13]	5.1 μM	5.1–500 μM	–	+	50 °C 2 h
Ninhydrin- CH_3COOH-H_3PO_4 [10]	Not shown	200–1800 μM	–	N-Terminal tryptophan peptides + C-Terminal tryptophan peptides -	100 °C 10 min
p-dimethylamino-benzaldehyde-H_2SO_4-$NaNO_2$ [25]	0.4 μM	0.4–20 μM	–	+	100 °C 10 min + 25 °C 15 min
Our method	100 μM	100–600 μM	–	–	37 °C 1 h + 25 °C 1 h

and thus the method cannot be applied to biological samples rich in indole compounds.

3.3. Free tryptophan detectability in complicated biological samples

With the established free tryptophan quantification chemistry in hand, we sought to test the application of HTA in real-world samples. To this end, we selected two mediums, which are commonly used for tryptophan fermentation, namely LB and M9-YE [22] with customized recipe. These two are complex medium with tryptophan from tryptone or yeast extract. We firstly measured their tryptophan content by HTA. As we expected, the concentration of free tryptophan detected in LB medium was much more than M9-YE. Then we measured the recovery rate of HTA in these two mediums by adding 100 μM tryptophan. Around 96% and 86% recovery rate was maintained in LB and M9-YE (Fig. 4a), preliminarily elucidating the robustness of our method to quantify concentration of tryptophan in complicated biological samples.

Tryptophan production based on microbial cell factory is a promising approach as a branch of upcoming bioeconomy [23], where strain breeding is one of the most important issues. Inspired by the extensive utilization of random mutant library screen strategy in microbial cell factory optimization [5,24], we further sought to test whether HTA can be adopted as a rapid quantification method for tryptophan titers after fermentation. This experiment was based on a previously constructed mutant library of *E. coli* tryptophan producers with diverse tryptophan production profiles in our laboratory (unpublished data). We used either HPLC or HTA to quantify their tryptophan titers after 24 h fermentation in M9-YE medium. The result of these two methods agreed well with each other (Pearson correlation coefficient = 0.93) (Fig. 4b), suggesting that HTA was suitable for free tryptophan quantification in complicated biological samples such as the fermentation broth. We replicated this experiment for two additional times and observed similar correlation between HTA method and HPLC, suggesting the reliability of HTA method. It should be noted that the absolute tryptophan concentration we obtained via HTA is bigger than HPLC (Fig. 4b), indicating a potential background signal detected by HTA in the fermentation broth. We tested this hypothesis by removing the TnaA enzyme in our protocol (add only KPbeta buffer without TnaA) and indeed observed background readouts, which might be due to substance(s) formed during fermentation interfering with the HTA chemistry. However, if we removed this background value in the data analysis, the correlation coefficient of quantified tryptophan titer between HIA and HPLC decreased to 0.74.

In spite of moderately decreased precision, this result showed that HTA could give the absolute quantification of tryptophan by background normalization. The impairment of quantification precision might be due to either the diverse background signals across the sample collection or the chemicals with impact on the accuracy of standard curve constructed in pure water. Even though, we argued that HTA, compared as HPLC, can be used as a fast and high-throughput method to give semi quantitative results when applying to complicated biological samples with similar background. The application scenario of HTA can be to rapidly identify the biological samples with outlined tryptophan concentration in contrast to the given reference sample (like the fermentation broth of the parent strain), which is the routine of many analytic efforts in food, environmental chemistry or biotechnology field. The impact of biological sample matrix on the accuracy of HTA can be determined by using HPLC or other separation based methods as gold standard, like in Fig. 4b.

4. Conclusion

We showed a colorimetric method to quantify the free tryptophan based on tryptophanase derived tryptophan-to-indole conversion and subsequent reaction with hydroxylamine to form pink product. This method exhibited linear quantification range from 100 μM to 600 μM and detection limit to 100 μM (signal:noise = 3:1). We verified the specificity of this method for free tryptophan using tyrosine, phenylalanine and two dipeptides (Ala-Trp as well as Trp-Gly) as competing chemicals. Thus, our method presented an important improvement that proteins in biological samples do not interfere with the assay in contrast to other separation-free colorimetric methods. Other metrics compared with these methods are summarized in Table 2. We confirmed the robustness of our method by quantifying free tryptophan concentration in complicated biological samples, such as microbe cultures. Typically, an individual investigator can complete the whole manipulation process in 2 h with simple equipment including multichannel pipettes and 96 well-plate readers, enabling the processing of hundreds of samples simultaneously. The tryptophanase can be obtained with a very high yield (5 mg/100 mL culture, correspond to 2500 reactions in the current protocol) and its activity can be also maintained in glycerol stock at −20 °C for at least two weeks (we observed partially maintained activity for six months after enzyme preparation). To make it easy to reproduce this method, we also provided a detailed protocol as Supplementary material in standard format. Based on these considerations, our method should be a convenient approach for rapid and specific quantification of free tryptophan for laboratories working in diverse fields such as medicine, microbiology, food chemistry or biotechnology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the

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