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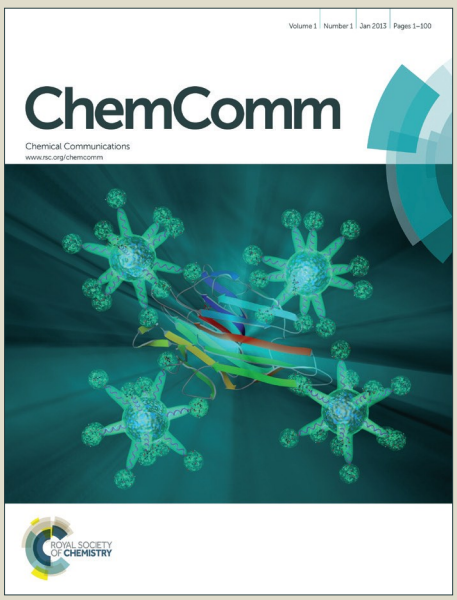

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ARTICLE TYPE

A novel, colorimetric method for biogenic amine detection based on arylalkylamine N-acetyltransferase

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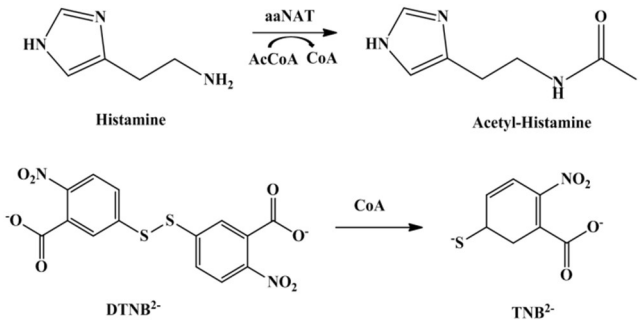
We developed a novel colorimetric method for rapid detection of biogenic amines based on arylalkylamine N-acetyltransferase (aaNAT). The proposed method offers distinct advantages including simple handling, speed, low cost, good sensitivity and selectivity.

Biogenic amines are basic nitrogenous compounds with at least one primary amine group, mainly derived from the decarboxylation of their precursor amino acids. [1] They are widely present in a variety of food products, such as milk, fermented vegetables, fish and fish products, beer, red wine, cheese, etc.[2] The presence of biogenic amines in food usually poses a public health concern, due to their physiological and toxicological effects. Histamine (HI), putrescine (PUT), cadaverine (CAD), tyramine (TA), tryptamine (TR), phenylethylamine (PEA), spermine (SPM), and spermidine (SPD) are considered to be the most important biogenic amines occurring in foods.[3] Of these, histamine is the most widely studied, due to its contributions to frequent outbreaks of food poisoning. Binding of histamine to receptors on cellular membranes can lead to headaches, low blood pressure, hypertension and digestive problems.[3] Putrescine, cadaverine, tyramine, phenylethylamine can inhibit histamine-detoxifying enzymes, thereby exacerbating the toxicity of histamine.[4,5] Tyramine can accumulate in the blood as a result of frequent intake of fermented and pickled food, leading to brain haemorrhage or heart failure.[3] Although putrescine, spermine, spermidine, and cadaverine have no adverse health effects, they may react with nitrite and produce volatile nitrosamines, which are classified as carcinogenic compounds. Moreover, elevated levels of certain biogenic amines may be found in food as a result of the use of poor quality raw materials, contamination and inappropriate conditions during food processing and storage, and have been used as indicators of food spoilage. It is reported that the total level of 1000 mg/kg (amine/food) poses potential threaten to human lives.[1] In fermented foods, the acceptable levels of histamine, tyramine, and phenylethylamine are 50-100 mg/kg, 100-800 mg/kg, and 30 mg/kg, respectively. In alcoholic beverages, the maximum concentration of histamine is no more than 18 μM.[1, 6] Considering the undesirable physiological effects of these compounds and their use as markers of food quality, it is important to monitor biogenic amines levels in foodstuffs and beverages.

The main obstacle for direct detection of biogenic amines lies in their structures, which contain no chromophoric or fluorophoric moieties. Biogenic amines are usually analysed by chromatographic methods, including high-performance liquid chromatography (HPLC),[7] thin layer chromatography (TLC),[8] gas chromatography (GC),[9] and capillary electrophoresis (CE).[10] For example, Konakovsky et al. employed HPLC to detect histamine in red wines with a limit of detection of 0.63 μM. This method required time-consuming sample preparation, including derivatization of the amines to form non-polar compounds (the highly polar amines tend to "stick" to the column and cause "memory effects").[11] Other chromatographic methods also suffer from costly, time-consuming procedures, and are not suitable for on-site detection. To overcome the time limitation, a variety of chromo- or fluorogenic chemosensors such as pyrylium compounds,[12] coumarin derivatives,[13] and fluorescent probes[14] have been developed for rapid detection of biogenic amines. For example, Lee *et al.* synthesized a molecular probe based on a coumarin derivative to determine biogenic amines in buffered aqueous solution by UV-Vis or fluorescence spectroscopy.[12] Nevertheless, the synthetic process of customized probe is also time-consuming, and this type of probe is not economical in practical applications.[13] Thus, it is desirable to develop a universal, rapid, direct method for "on the spot" detection of biogenic amines.

It has been reported that arylalkylamine N-acetyltransferase (aaNAT), which is involved in melatonin formation, sclerotization, and neurotransmitter inactivation, employ serotonin and other endogenous amines as substrates and catalyse the transacetylation of acetyl coenzyme A (AcCoA).[15] To the best of our knowledge, the capacity of aaNAT to acetylate biogenic amines with a characteristic spectroscopic signature has not been applied to the analysis of these compounds. As shown in Scheme 1, taking histamine as a model, aaNAT specifically recognizes the active amino group of histamine, a similar structure shared by all biogenic amines, for transacetylation, in which an active hydrogen atom on the amino group is replaced by an acetyl group (CH_3CO-) from AcCoA, yielding acetyl-histamine. The free thiol group of the remaining CoA can react rapidly with an aromatic disulphide, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB).[16] DTNB is the famous Ellman's reagent that has been widely applied for spectrophotometric measurement of free thiols or accessible protein thiols since 1959.[17] Applications of DTNB include assays of the thiol content of proteins,[18] analysis of acetylcholinesterase,[19] and determination of the acetyltransferase activity.[16] Ellman's reagent

is water soluble at neutral pH and its reaction with free thiols is sensitive and rapid, to release an equivalent of a highly chromogenic, yellow product 2-nitro-5-thiobenzoate anion (TNB^{2-}) as well as a mixed disulfide.[20] TNB^{2-} exhibits intense light absorption at 412 nm, which can be conveniently measured by UV-vis spectroscopy, and the absorbance is directly proportional to the amount of biogenic amine of histamine present in the tested sample.



Scheme 1. Schematic illustration of the proposed method of histamine detection based on aaNAT.

Mehere and co-worker isolated aaNAT (EC 2.3.2.87) from *Aedes aegypti* mosquitoes.[15a] Because it is difficult to raise and process mosquitoes to obtain aaNAT directly, we first focused on finding of a suitable microbial expression system for aaNAT. *Escherichia coli* (*E. coli*) is a mature bacterial expression system and would be a good alternative,[21] due to its short generation time and ease of plasmid manipulation. However, as a prokaryotic expression system, *E. coli* suffers from codon usage bias that restricts the expression of a heterogonous gene. The initial gene of aaNAT coming from *Aedes aegypti* mosquitoes may hamper the acquisition of high yield of recombinant protein. Thus, we altered the DNA sequence of aaNAT (*aanat* gene) so that the codon bias was consistent with that from *E. coli* (See Fig. S1 in ESI). As shown in Fig. S2, the recombinant protein was abundantly expressed in the host strain of *E. coli*, indicating that the optimization of *aanat* gene codon was successful.

Following acquisition of protein aaNAT, we investigated the sensitivity of the proposed method for biogenic amines detection. Histamine is a powerful biologically active chemical and can exert many responses within the body, thus it was selected as the model. A series of histamine standards (0, 5, 10, 50, 100, 250, 500, 750, 1000, and 1200 μM) was added to the reaction mixtures containing aaNAT (0.5 μM), AcCoA (1 mM), and DTNB (2 mM), in HEPES buffer (50 mM, pH 7.5) in a total volume of 100 μL. Reactions were monitored at room temperature by UV-vis spectroscopy at 412 nm every 30 s for 20 min. As shown in Fig. 1A, a gradual increase in the absorbance at 412 nm was clearly observed with an increase in the concentration of histamine from 0 to 1200 μM, and the color of the reaction mixture gradually changed from colorless to dark yellow. The initial rate (v_0) of acetylation was determined for a constant concentration of aaNAT (0.5 μM) and histamine concentrations between 0 and 7000 μM. The data were fitted to a Michaelis–Menten kinetics equation as shown in Fig. 1B with a best-fit equation: v_0 = 16.27[HI] / (1769 + [HI]). The maximum acetylation velocity (v_{max}) and Michaelis constant (K_m) were calculated to be 17032 ± 567.1 nM min^{-1} and 1921 ± 147.7 μM, respectively. The inset in Fig. 1B demonstrates that a relatively simple spectrophotometer can achieve a detection limit of 5 μM. In addition to histamine, the proposed method was also applied to detect tyramine, and phenylethylamine, which both exhibit significant physiological and toxicological effects. As shown in Fig. S3 and Fig. S4, Michaelis–Menten kinetics equations were obtained for tyramine, and phenylethylamine, respectively. In addition, linear equations ΔA = 0.0014[TA] + 0.0033, R^2 = 0.9999 (0-1000 μM) and ΔA = 0.0019[PEA], R^2 = 0.9999 (0-750 μM), were also obtained, respectively.

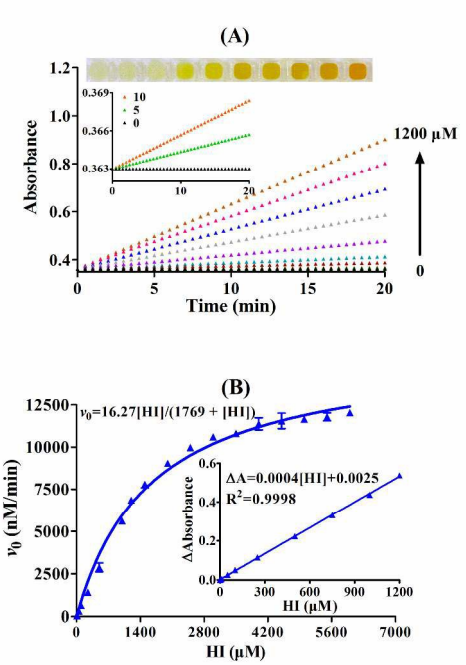


Fig. 1 (A) Plots of absorbance at 412 nm vs time for aaNAT (0.5 μM) treated with histamine (HI) targets at different concentrations (0, 5, 10, 50, 100, 250, 500, 750, 1000, and 1200 μM). Inset: magnification of the plot at the concentrations of 1, 5, and 10 μM. (B) Plot of initial velocity vs concentration of histamine from 0 to 7000 μM at a constant amount of aaNAT (0.5 μM) at 25°C. Inset: the linear plot of the change of absorbance in 20 min vs histamine concentration in the range of 0-1200 μM

To test the specificity of the proposed method, a panel of related compounds of biogenic amines, amino acids, and aminoglycoside antibiotics were examined under the same conditions. These compounds included phenethylamine (PEA), tyramine (TA), histamine (HI), glycine (G), valine (V), leucine (L), phenylalanine (F), proline (P), tyrosine (T), arginine (R), tryptophan (W), lysine (K), histidine (H), amikacin (AMK), kanamycin (KAN), and neomycin (NEO). Fig. 2 shows the absorbance differences at 412 nm after 20 min reaction time. The biogenic amines produced an obvious change in the absorbance, while there was no detectable change in the presence of other amino acids. The results clearly demonstrate that as a biological enzyme, aaNAT can specially recognize biogenic amines and catalyse their acetylation in the presence of AcCoA, even in the presence of amino acids and aminoglycoside antibiotics having similar structures.

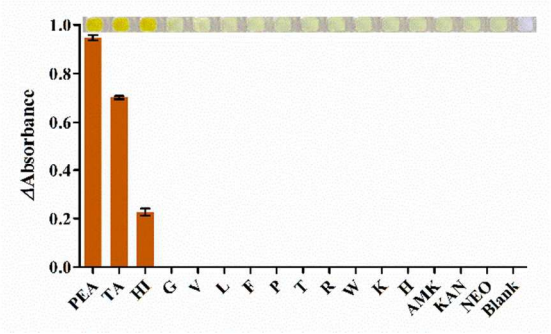


Fig. 2 Bars represent the absorbance change for 20 min in the presence of biogenic amines (500 μM), or amino acids (500 μM). From left to right: phenethylamine (PEA), tyramine (TA), histamine (HI), glycine (G), valine (V), leucine (L), phenylalanine (F), proline (P), tyrosine (T), arginine (R), tryptophan (W), lysine (K), histidine (H), amikacin (AMK), kanamycin (KAN), neomycin (NEO), and blank sample (no biogenic amines or amino acids added).

Besides sensitivity and selectivity, the proposed method should be applicable to food samples in a variety of scenarios. We next detected biogenic amines in white wine and milk sample (see ESI for detailed pre-treatment procedures). The pre-treated white wine and milk sample had no absorption, indicating no detectable biogenic amines present. Then, we further carried out spike-in experiments by adding histamine, tyramine, and phenethylamine with various concentrations to white wine and milk samples, and detected by our proposed method. Based on the above linear equations, the results obtained were in good agreement with the added amounts. These results are summarized in Table S1 for histamine, Table S2 for tyramine, and Table S3 for phenethylamine, in which the recoveries were in the range of 97.05~119.00 %. In addition, we also apply the proposed method to detect histamine in complex biological sample of the cell culture supernatant (see ESI). The results obtained by our method were in good agreement with those from the commercial ELISA kit (Fig. S5). These results clearly indicated that the proposed method promises good accuracy and reliability in the practical applications.

In conclusion, we have applied catalysis by genetically modified aaNAT for colorimetric detection of biogenic amines. Compared to other methods, the proposed assay offers distinct advantages. First, this method is a simple enzymatic reaction, in which aaNAT transfers an acetyl group from AcCoA to biogenic amines in the presence of DTNB, resulting in formation of TNB^{2-}, which absorbs strongly at 412 nm. Second, the detection cost is not high due to the employment of a novel biological enzyme. Traditional chromo- or fluorogenic methods require synthesis of complex or toxic chemical probes, while overexpression of this chromosomally encoded acetyltransferase in *E. coli* is convenient and economical. In addition, the catalytic activity of aaNAT remains stable after long-term storage of the stock solution, which indicates the feasibility of developing commercial detection kits for detection of biogenic amines in the future. Third, the response of this assay takes place with 20 min, which is superior to the performance of most of the existing detection methods. Fourth, the method provides good sensitivity towards histamine with a detection limit of ~5 μM, and good selectivity to distinguish biogenic amines from their precursors and similar compounds. Fifth, high recovery data in real sample detection promises reliability in practical application. Combining these significant features, we believe that the proposed colorimetric assay has great potential as a powerful tool for the analysis of biogenic amines in the fields of food safety and medical diagnosis.

Notes and references

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