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Spectrophotometric detection of histidine and lysine using combined enzymatic reactions

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ABSTRACT

An amino acid-sensing system with absorption spectrophotometric detection was developed. To ensure specific recognition of each amino acid, aminoacyl-tRNA synthetases were employed and the concentration of NADH produced by way of several enzymatic reactions was measured. Using this detection system, from 1.5 to 55 μM of histidine and from 15 to 95 μM of lysine could be measured selectively in HEPES-KOH buffer (pH 8.0).

1. Introduction

Amino acids are important compounds constituting the living body; thus, their analysis in biological fluids can provide relevant information [1,2]. Measurements of amino acids are also informative in determining the freshness and nutrition of foods [3,4]. Pre-column labeling of amino acids with fluorescence derivatives or post-column labeling with ninhydrine for high-performance liquid chromatography (HPLC) [5,6] are the conventional methods for amino acid detection. These analytical methods are effective for the measurement of free amino acids, but have several drawbacks, including the extensive time and costs involved.

Biosensors are widely applied in clinical, environmental, and food analyses and enable both rapid and easy detection [7–9]. Although it has been reported that an amino acid sensor for lysine using lysine oxidase has been fabricated [10] and that a sensor for phenylalanine was fabricated with phenylalanine dehydrogenase as the molecular recognition element [11], the sensitivity and the selectivity of these sensors do not allow their clinical use. We have reported generation of tyrosine-sensors using tyrosyl-tRNA synthetase as the molecular recognition element; tyrosine could be measured selectively, but the measurable range was at the level of several hundreds of micromoles and was also not suitable for clinical analysis [12,13].

However, aminoacyl-tRNA synthetases (ARSs) are involved in the biosynthesis of peptides and proteins in the human body [14–18], and should allow precise recognition of the corresponding amino acids. In the present study, we investigated an amino acid-sensing method using histidyl-tRNA synthetase (HisRS, a histidine-specific ARS) and lysyl-tRNA synthetase (LysRS, a lysine-specific ARS), as the molecular recognition element. In this method, the conversion of NAD^+ to NADH by way of several enzymatic reactions was measured spectrophotometrically by monitoring the absorbance change at 340 nm. The quantitativity and selectivity of each sensor were also evaluated.

2. Experimental

2.1. Reagents

Amino acids, luminol, dithiothreitol, triethanolamine hydrochloride, potassium hydroxide, hydrochloric acid, and sodium hydroxide were purchased from Wako Chemicals (Osaka, Japan). Inorganic pyrophosphatase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglyceric phosphokinase, glyceraldehyde-3-phosphate, NAD^+, adenosine-5′-triphosphate, adenosine-5′-diphosphate, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyruvate oxidase was purchased from Asahi Kasei Corp. (Tokyo, Japan). ARSs were commissioned from Takara Bio Inc. (Shiga, Japan). *N,N*-Bis(2-hydroxyethyl) glycine was purchased from Dojindo Laboratories (Kumamoto, Japan). The chemicals were commercial reagents of the highest grade and were used without further purification. The purity of histidine was 98%, that of lysine was 97%, and that of other amino acids was 99%.

HEPES-KOH buffer (22 mM) was prepared as follows: HEPES (5.24 g) was dissolved in 800 mL of distilled water, the pH adjusted with 1.0 M KOH, and the volume brought to 1.0 L with distilled water. In the same manner, 22 mM bicine-NaOH buffer was prepared by

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dissolving *N,N*-bis(2-hydroxyethyl) glycine (3.59 g) in distilled water, and the pH adjusted with 1.0 M NaOH, and triethanolamine-HCl buffer was prepared by dissolving triethanolamine hydrochloride (4.01 g) in distilled water and adjusting the pH with 1.0 M HCl, and the volume brought to 1.0 L with distilled water.

2.2. Enzyme reaction and assay

Histidyl-tRNA synthetase (HisRS, 79 μg mL^{-1}), 2.0 mM ATP, 5.0 mM magnesium chloride, 450 mM potassium chloride, and 0.9 mM dithiothreitol were dissolved in 22 mM HEPES-KOH buffer (pH 8.0) and used as reaction buffer. An aliquot (10 μL) of each amino acid solution, ranging in concentration from 0 to 100 μM, was added to 90 μL of the reaction buffer, and incubated for 30 min at 40 °C. To the reaction mixture, 100 μL of a solution containing 1.0 U mL^{-1} inorganic pyrophosphatase, 2.0 U mL^{-1} glyceraldehyde-3-phosphate dehydrogenase, 1.0 U mL^{-1} 3-phosphoglyceric phosphokinase, 1.2 mM ADP, and 1.2 mM NAD^+ in 22 mM HEPES-KOH buffer (pH 8.0) was added, and the samples incubated for 60 min at room temperature. The rate of NADH formation was measured by the change in absorbance at 340 nm using a microplate reader (Synergy 4, BioTek Instruments, Inc., Winooski, VT, USA). The data shown represent average values of 3 measurements and the standard deviation is indicated by error bars.

For lysine sensing, lysyl-tRNA synthetase (LysRS, 180 μg mL^{-1}) was used as the recognition element, and the enzyme reaction and assay were conducted in the same manner as for histidine sensing, except with the addition of LysRS instead of HisRS addition.

2.3. Amino acid analysis

A Hitachi high speed amino acid analyzer Model L-8900BF (Tokyo, Japan) was used in the analysis. L-8500-PF-KIT (Mitsubishi Chemical Company, Tokyo, Japan) was used as eluting buffer, and L-8500 Set (Wako, Osaka, Japan) was used as ninhydrin reagent. A packed column (2622SC; 60 mm × 80 mm i.d.) produced by Hitachi, Ltd. was used as separation column. The analyzer was operated using the supplied analysis program.

3. Results and discussion

Each ARS reacts with a corresponding amino acid, releasing inorganic pyrophosphate [Eq. (1)]. Inorganic pyrophosphate is hydrolyzed in the presence of inorganic pyrophosphatase, releasing phosphate [Eq. (2)]. Phosphate reacts in the presence of NAD^+, glyceraldehyde-3-phosphate, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglyceric phosphokinase, producing NADH [Eq. (3)] [19,20]. In the present study, the rate of NADH formation was measured by assessing the absorbance change at 340 nm using a microplate reader.

3.1. Histidine sensing

A histidine-specific enzyme, HisRS, was used for histidine recognition, and the reaction conditions evaluated. Fig. 1 shows the calibration curve for histidine sensing. The horizontal axis represents the initial concentration of histidine, and the absorbance change at 340 nm is represented by the vertical axis. The absorbance change increased in response to histidine addition, and a range of histidine concentrations, from 1.5 to 55 μM, could be measured ($r = 0.986$), with a detection limit of 1.5 μM, at a signal-to-noise ratio of 3. Spectral scans of the detection of NADH using the HisRS reaction is shown in Fig. 2. The HisRS reaction in the absence of histidine (dotted line) and with 20 μM histidine (solid line) is shown. As expected, the absorbance intensity at 340 nm was increased in the presence of histidine in the HisRS reaction.

The effects of pH and buffer conditions on the ARS enzyme reaction were also tested (Fig. 3). As reaction buffers, HEPES-KOH buffer with a pH ranging between 6.8 and 8.8, bicine-Na buffer with a pH ranging between 8.0 and 8.8, and triethanolamine-Cl buffer with a pH ranging between 8.0 and 8.8 were evaluated; these results are shown in Fig. 3. The absorbance increased along with an increase in pH, up to a pH value of 8, when it plateaued between pH 8.0 and 8.6. No difference was seen with the different types of buffer.

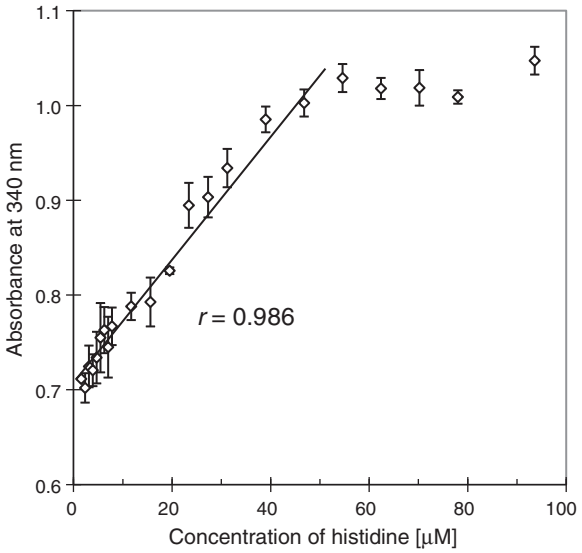

Fig. 1. Calibration curve for histidine in the histidine sensing method.

The effect of the concentration of the reaction buffers (between 22 and 70 mM for HEPES-KOH buffer (pH 8.0)) on enzyme response was tested and no marked differences were observed (data not shown). Thus, the buffer conditions chosen were 22 mM HEPES-KOH buffer at pH 8.0.

As our previous study, ARS reacted time-dependently. Thus, the ARS reaction time was fixed at 30 min and the subsequent enzyme reaction time with the mixture of inorganic pyrophosphatase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglyceric phosphokinase was examined. As shown in Fig. 4, the absorbance intensity for histidine increased in a time-dependent manner. Hence, a reaction time of 60 min was chosen for the subsequent experiments.

A selectivity test of HisRS was conducted with the addition of each of 20 amino acids at a concentration of 50 μM, and without amino acids as reference (Fig. 5). A marked change in absorbance was observed for

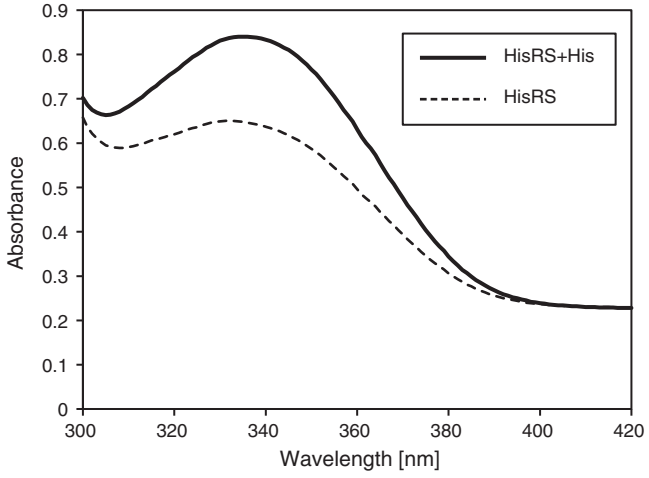

Fig. 2. Spectral scans of the detection of NADH with the Histidyl-tRNA synthetase (HisRS) reaction. HisRS reacted with 50 μM histidine (solid line) and the absorbance at 340 nm shifted increased compared to the reaction lacking histidine (dotted line).