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Determination of the Enzymatic Activity of S-Adenosylhomocysteine Hydrolase in Parasites Using HPLC

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ABSTRACT

S-Adenosylhomocysteine hydrolase, also known as S-adenosylhomocysteine synthase or adenosylhomocysteinase, is one of the sulfur-containing amino acid metabolic enzyme, which can be found in the parasite *Giardia intestinalis*. In this study, a HPLC analysis method was developed for the measurement of its activity. Such method was simplified as much as possible in order to allow for short analysis time and convenient operation. As a result, adenosine, homocysteine, and adenosylhomocysteine, which correspond to the substrate and the product of the enzymatic reaction, could be simultaneously detected spectrophotometrically within approximately 10 min in the order of 0.01-1 mM ($r \geqq 0.999$).

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INTRODUCTION

Among all infectious diseases, parasitic infections were mostly eradicated in Japan with the spread of sewers and improvement of hygiene after World War II[1)]. However, recently the consumption of pesticide-free and organically grown vegetables has increased due to the healthy food boom, together with the consumption of bizarre food items, breeding of various types of pets, and overseas travels by plane within a short time. As a consequence, some of the previously prevalent parasites have been reintroduced and new types of diseases have emerged in developing countries[2].

In recent domestic cases in Japan, tap water was found to be contaminated by Cryptosporidium protozoa, which is known to cause opportunistic infections in immunocompromised patients like those affected by AIDS (Class 4 of the Infectious Diseases Control Law). Generally, there is a poor understanding of many parasitic diseases currently emerging in Japan as well as their related conditions, which may lead to delays in treatment, and as a result, additional complications[1)]. Therefore, it is unclear whether such parasitic diseases may become widespread and cause fatal conditions in the future. Alike other countries, Japan should be aware of the global trend of infectious diseases and be prepared for potential health threats. A timely strategy is highly desirable that could permit to investigate the causes of individual parasitic diseases, and particularly identify and understand the infection routes, in order to develop effective treatments. This would allow not only for early prevention but also acting quickly when an infection is confirmed, thus preventing the worsening of the disease and spreading in the surrounding region.

Currently, our work focuses of finding a new enzymatic reaction system involved in the metabolism of sulfur-containing amino acids, for which either the reaction conditions differ between humans and parasites or the specific reaction pathway is present only in parasites, with the aim of developing new drugs against parasitic diseases, especially those caused by protozoa[3-6)]. With regard to the new enzyme that has been discovered, we investigated the reactivity, such as the optimal reaction conditions and activities that are characteristic of the enzyme, and intend to further explore the development of inhibitors.

S-adenosylhomocysteine hydrolase, which is also known as S-adenosylcysteinehomocysteine synthase or adenosylhomocysteinase, has already been cloned from *Giardia intestinalis* gene using molecular biology techniques and analyzed by Tokoro and co-workers[6-8)]. In this study,

we developed a simple HPLC based method for the measurement of the enzymatic activity as a tool to investigate the reaction characteristics of this enzyme. This enzyme synthesizes adenosylhomocysteine using adenosine and homocysteine as substrates. Similar enzymes exist in the human body, however, they are known to catalyze only the reverse degradation reaction (Fig. 1)[9]. A survey of several conditions allowed an establishment of a simple measurement method.

METHODS

Strategy

In this study, we aimed to develop an HPLC method that would allow to determine the activity (units) of an enzyme, optimum reaction conditions, and potential inhibition of the enzymatic reaction by drug. Generally, an efficient HPLC analysis method requires: 1) good separation of proteins, lipids, carbohydrates, and drugs that are expected to be contaminated with a target enzyme, and 2) high sensitivity according on the physical properties of the target compounds (absorption maximum wavelength, etc.). In addition, it is essential to separate from the target compounds other potential contaminants such as foods and biological samples.

However, in order to achieve this goal, it is necessary to establish a method as simple as possible. For example, a) a gradient elution should be not performed using a simple system configuration, b) the analysis should be completed in a short time to allow for the analysis of a large number of samples, and c) the detection should be performed only on the basis of the physicochemical properties of the compounds, which most desirably should not to be derivatized. In our case, the amount of contaminants was not very large unlike general real samples. Therefore, we decided to use only one liquid pump within the analytical system and perform an isocratic elution (Fig. 2). The employed system was simply obtained by adding a column oven to a minimal HPLC system. Other than that, the system configuration was very simple. A column oven was initially added because it was assumed that the separation changed depending on the column temperature. However, when the analysis was conducted at room temperature different peaks were observed corresponding to the three target substances, therefore eventually it was not necessary to equip the system with an oven.

This is because we aimed at the establishment of a technique that merely analyzes enzyme

reactants, and only samples containing enzymes, reagents during reaction, substrates, and products. Since it was decided to employ conditions that could be used even in the presence of a relatively high concentration of the substrate and low analytical sensitivity, the most popular ultraviolet spectrophotometric detector for HPLC was used.

The progress of the enzymatic reaction itself can be monitored by detecting either an increase in the product (adenosylhomocysteine) or a decrease in the substrates (adenosine and homocysteine). In order to improve the reliability of the results, we decided to analyze the variations of both substrates and products simultaneously. In addition to methods based on flow analysis systems such as HPLC, a batch method has also been described[10,11]. In this case, it is somewhat difficult to follow the time course of the reaction, as a main drawback of this method consists in the difficulty to perform bidirectional measurements simultaneously; thus, only a single component, *i.e.*, either the substrate or product, can be detected at a time. Therefore, appropriate results cannot be obtained if the measurements are disturbed by the presence of drugs. In fact, this method is better for obtaining reliable data since no interferences between different substances occur.

Here, only the basic conditions established for the developed HPLC method were described, while a summary of the properties of the enzyme will be reported elsewhere.

Reagents

Adenosine and homocysteine (special grade) were manufactured by Wako Pure Chemical Industries (Osaka, Japan). Adenosylhomocysteine was purchased from Funakoshi Co., Ltd. (Tokyo, Japan) and used without further purification. A 1 M aqueous solution of each substance was prepared, frozen, and stored at -20°C. These samples were allowed to thaw in an ice-bath before use, further diluted with water up to the required concentration to provide standard solutions for HPLC analysis. The phosphate salts and methanol (special grade) used as eluents for HPLC were obtained from Wako Pure Chemical Industries. With regard to the other reagents, commercially available special grade products were used.

The S-adenosylhomocysteine hydrolase used in this study was prepared using a cell-free culture system. The method employed can be briefly described as follows. RNA was obtained from *Giardia intestinalis* parasites derived from animal feces using a commercially available extraction kit. RNA was then transcribed into DNA using a reverse transcriptase, and then the

gene sequence corresponding to the target enzyme was amplified by PCR using an appropriate DNA primer. It was then cleaved by restriction enzymes, introduced into a plasmid, and the protein was expressed in *E. coli*. *E. coli* cells successfully transformed was selected, cultured to obtain a sufficient number of cells, and the enzyme was recovered. The crude extract (containing the desired enzyme) was frozen and stored at −80°Cafter addition of glycerol. Adjusting the protein concentration to 1 μg/mL was subjected just before the enzymatic reaction. All the water used was purified over 18 MΩ·cm.

Apparatus

The HPLC apparatus used consisted of a pump (LC-6A, Shimadzu; Kyoto, Japan; 1.0 mL/min), manual injector (Rheodyne 8125 type, Idex Health & Science; Oak Harbor, WA, USA; connected with a 100 μL loop), reversed-phase ODS column (TSKgel Super-ODS, 4.6mm i.d. x 100mm long; Tosoh, Tokyo), column oven (CTO-2A, Shimadzu; 40°C), ultraviolet spectrophotometric detector (SPD-6A, Shimadzu), and recorder (Chromatopack C-R3A, Shimadzu). The usage conditions were only those described, and no other conditions were considered in this study.

A spectrophotometer U-5100 (Hitachi; Ibaraki, Japan) was used. Water was purified with Elix 3/Element A10 (Merck-Millipore; Billerica, MA, USA).

Sample pretreatment

For the sample pretreatment, 900 μL of a mixed solution of buffer (10 mM phosphate buffer, pH 7.0) and 1 mM substrates (adenosine and homocysteine) was added to 100 μL of an S-adenosylhomocysteine hydrolase solution and reacted at 37°C. After the reaction was completed, methanol was added as protein denaturant, followed by filtration with a membrane filter (Mirex HA, Merck-Millipore; pore size 0.45 μm). The resulting filtrate was used as sample for HPLC.

RESULTS AND DISCUSSION

Measurement wavelength

In this method, the components to be measured correspond to the substrates and product of the enzymatic reaction. When the enzymatic reaction is prolonged and the concentration of the substrates is high, it is possible to increase the amount of the product as the reaction

progresses, which results in an increase of the peak height. On the other hand, the amount of product can be increased by increasing the amount of enzyme. Therefore, it is not always necessary to perform high sensitivity measurements. In this study, an ultraviolet spectrophotometric detector was used, and the same wavelength was used for each compound and time. As preliminary tests, the absorption spectra of the three compounds (adenosine, homocysteine, and adenosylhomocysteine) were recorded using a spectrophotometer, however, no significant absorption maximum was observed in the 190-350 nm range. The absorbance decreased as the wavelength increased. Among the three compounds, homocysteine exhibited the lowest absorbance due to the absence of an aromatic ring in its structure. Therefore, the measurement wavelength was selected to be 195 nm, which corresponds to a relatively high absorbance of cysteine and at the same time it is a measurable wavelength for the detector.

Column type

In this study, a reversed-phase TSKgel Super-ODS column bearing octadecyl groups was used as HPLC column. Among the three compounds of interest, homocysteine contains a cysteine residue with a carboxylic group, thus forming an anion at high pH. Conversely, adenosine possesses a nitrogen-containing aromatic ring with an amino group and forms a cation at low pH. Adenosylhomocysteine is the product of a dehydration reaction between the primary hydroxyl group of the ribose of adenosine and the sulfhydryl group of homocysteine, and it is an amphoteric substance as it exhibits both anionic and cationic properties. On the basis of this knowledge, it was assumed that the simultaneous separation of these substances under anion or cation exchange conditions could be difficult. On the other hand, the use of a reversed-phase column could allow for a significant change in retention time depending on the concentration of the organic solvent, making the simultaneous detection of anions and cations possible by adjusting the pH and ionic strength of the buffer solution. Moreover, the use of an organic solvent could also suppress ionization. Therefore, the measurements were conducted only under these conditions. Among all the ODS columns available, only the above-mentioned column, which was assumed to permit separation in a short time, was used for the measurements. Compared to other columns, this column had a smaller particle size of the packing material and a larger surface area per unit mass, therefore it was assumed that the number of theoretical plates was high and an easy separation could be achieved in a short time.

HPLC eluent composition

The eluent composition affects the peak shape as well as the separation of the specific compounds. If the composition of the solution in which an enzymatic reaction is performed differs from the composition of the HPLC eluent, peak leading or tailing can occur. Therefore, the phosphate buffer and methanol used for the pretreatment of the samples were also used as eluent components. Prior to optimization, no noticeable interference between the coexisting substances was observed, and a chromatogram similar to that of the standard product was obtained (figure is not shown). The pH of the phosphate buffer and methanol concentration were changed in order to improve the resolution and peak height. As a result, an optimal separation was obtained when a 10 mM phosphate buffer (pH 7.0)/methanol (10:90) mixture was used as eluent.

Under these conditions, the concentrations and peaks of adenosine, homocysteine, and adenosylhomocysteine showed a good linear relationship with a correlation coefficient r ≧ 0.995 in the 0.1-1 mM range for both the peak height and peak area. It was found that the quantification was possible and that the adenosine and homocysteine concentrations decreased as the reaction progressed, whereas the adenosylhomocysteine concentration increased, leading to a quantitative conversion.

Measurements of a real sample

It was observed that the concentration of adenosine, homocysteine, and adenosylhomocysteine changed with the enzymatic reaction time. As a result of changing the amount of enzyme protein or the time interval, it was found that the generation of adenosylhomocysteine increased almost proportionally within 5 min to 1 h. Therefore, such method could be practically used for enzymatic activity measurements when target compounds are known. Even in the presence of other enzymes, many compounds have the ability to absorb light in the ultraviolet region. In similar experiments, since large amounts of contaminants cannot be introduced into the HPLC apparatus, it is not necessary to determine complicated separation conditions. When a similar pretreatment method is used, the proposed method can be applied to other enzymes by establishing suitable methanol content, buffer concentration, and pH conditions. In this experiment, a manual injector was used and the deproteinized sample was applied to the HPLC system. However, for more recent columns, it is possible to introduce samples with high protein concentrations directly into the HPLC. By

adding an auto injector (an instrument that can constantly inject a sample into the HPLC apparatus) to the system configuration, it is possible to calculate the reaction rate with a simpler operation, thus achieving a further improved accuracy.

CONCLUSION

In this study, the enzymatic activity of S-adenosyl homocysteine hydrolase was measured by HPLC. The pretreatment and analysis method after the enzymatic reaction were based on simple procedures. Therefore, it is anticipated that such method could be also employed to perform similar measurements for other enzymes upon optimization of the separation conditions of the target compounds.

In general, in order to effectively employ the HPLC technique, it is important to determine optimal experimental conditions, and therefore select a suitable column, eluent composition (organic solvent + buffer), and detection wavelength. In summary, A) unless all the substances to be measured can be converted into anions or cations, reversed-phase ODS columns should be considered first rather than ion exchange columns; B) the content of the organic solvent in the eluent system is useful for adjusting the detection time of the entire target substance (the higher is the content, the shorter is the elution time). However, since all substances exhibit either an extended or shortened but similar detection times when the separation is insufficient, a further examination of the buffer solution is necessary; C) the concentration of the buffer in the eluent system can influence the ionic strength, and the pH can change the ionization of the compound, thus regulating the strength of the retention to the column. Since this can vary for each target substance, this property can be exploited for achieving separation; D) if leading and tailing of the chromatogram peaks occur, it is better to use the same reagents for both pretreatment and HPLC analysis. For example, in this study, a phosphate buffer and methanol were used for both pretreatment and analysis stages. E) Up to D) these are conditions for achieving separation and short-time detection. In the case it is necessary to consider the analytical sensitivity, the wavelength adjustment shall also be considered. The molar extinction coefficient (the response value of the detector when a standard solution of a target substance is passed without a column attached, or the value measured with a spectrophotometer) or peak height (detector response value after column separation under appropriate conditions) can be obtained. To this aim, it is necessary to determine which compound should be measured with high sensitivity (or whether the same sensitivity is sufficient) and select the measurement wavelength accordingly. The study

described above is based on fundamental HPLC conditions. The examination of these conditions is based on the premise that the amount and quality of coexisting substances are not significantly different from those of the environment and biological samples, without almost no interference taking place.

In the future, we plan to use this method to investigate the properties of S-adenosylhomocysteine hydrolase (enzyme activity, inhibitory effects by drugs, etc.).

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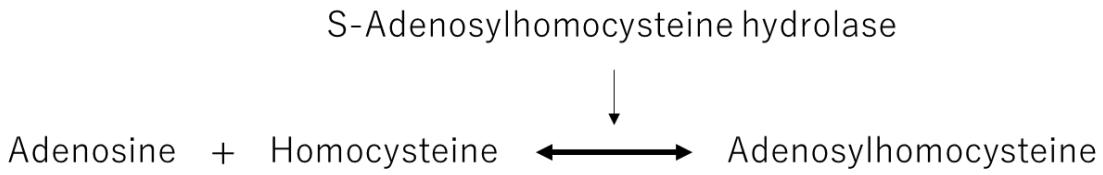

Figure No. 1: Schematic representation of the reactions catalyzed by S-adenosyl homocysteine hydrolase. The reaction pointing to the right side occurs inside the parasites, while the one on the left takes place in the human body

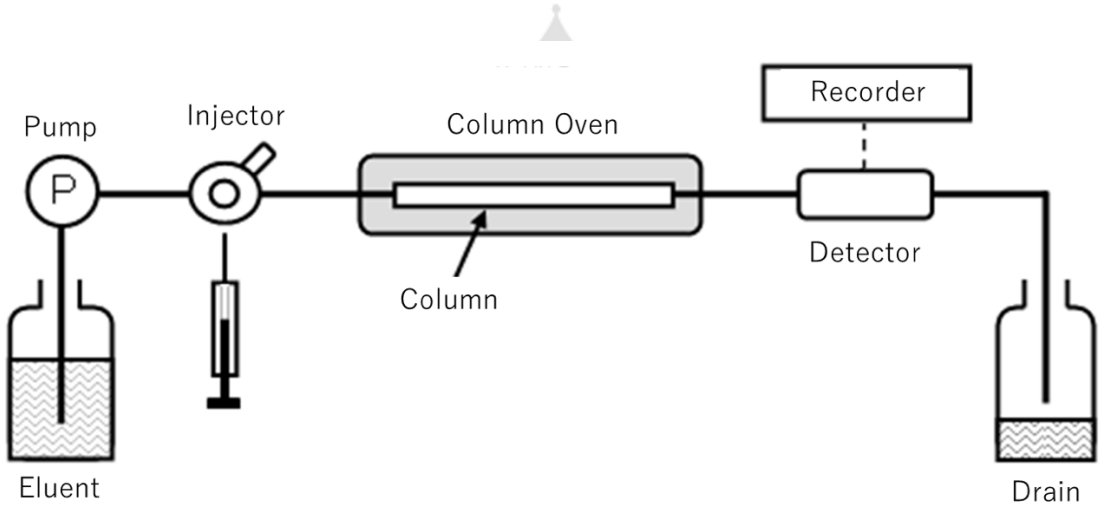

Figure No. 2: Schematic diagram of the HPLC system used in this study

A simpler configuration of this system can be obtained by removing the column oven from the equipment. An ultraviolet spectrophotometer serves as detector.