Novel Histamine Measurement by HPLC Analysis Used to Assay Histidine Decarboxylase Inhibitory Activity of Shoyuflavones from Soy Sauce

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Received January 13, 1998

An easy and highly sensitive method for measuring histamine by HPLC analysis coupled with precolumn derivatization was established. The amino group of histamine was completely colorimetrically labelled with 4-N,N-dimethylamino-azobenzene-4′-isothiocyanate (DABITC) in the presence of sodium bicarbonate at 90°C for 5 min. The derivative was sensitively and easily analyzed by HPLC on a Cosmosil 55S column using CHCl₃/N,N-dimethylformamide/H₂O (210:90:4) containing 0.4% acetic acid. Using the established method, histidine decarboxylase (HDC) inhibitory activities of three tartaric acid isoflavone derivatives, named shoyuflavones, isolated from soy sauce were examined in vitro by measuring the histamine produced by HDC. They showed intense inhibition of the activities of HDC from both mouse mastocytoma P-815 cells and Clostridium perfringens.

Key words: isoflavone; histamine; histidine decarboxylase; 4-N,N-dimethylamino-azobenzene-4′-isothiocyanate; histamine synthesis inhibition

Fig. 1. Structures of Shoyuflavones A, B and C from Fermented Soy Sauce.

From chemometric pattern recognition analysis of HPLC profiles of soy sauce, significant roles of three tartaric isoflavone derivatives, named shoyuflavones A, B, and C, respectively, and daidzein (Fig. 1) for differentiating soy sauce from various manufacturers and different starting materials were found. Coward et al. reported that fermented soybean products such as soy sauce, miso, and temph generally contain isoflavone β-conjugates and aglycones derived from soybeans. However, the three shoyuflavones may be specific to fermented soy sauce because, until now, they have not been found in soybeans or any other soy products. Although the biosynthesis mechanism has not been unveiled, they seem to be produced from isoflavone aglycones during the fermentation of soy sauce.

Recently, studies about histamine have been increasing because of its significant physiological roles as a mediator of inflammation, allergy, gastric acid secretion, and neurotransmission. Analytical methods for histamine in biological fluids have mostly been based on Shore’s method, in which fluorescence labelling using o-phthalaldehyde (OPA) is used. Although several attempts at HPLC analysis of histamine with derivatization by OPA, phenylisothiocyanate, or dansyl chloride have been reported, these methods cannot be used easily because of their troublesome sample cleanup and requirement for expensive columns. Moreover, these reagents used and the resulting derivatives in such methods are very unstable. Therefore, developing a easy, highly sensitive, and economical method for measuring histamine has been required. Chang and Creaser reported that 4-N,N-dimethylamino-azobenzene-4′-isothiocyanate (DABITC) stably formed different colored products with amino alcohols and amino acids, allowing identification of such compounds on TLC. Their results indicated us that the derivatization with DABITC and the following HPLC analysis should be useful for measuring determining histamine.

Inhibition of the L-histidine decarboxylase (HDC, EC 4.1.1.22) activity by several flavonoids has been reported. Especially, among these flavonoids, (++)-catechin has a low acute toxicity and inhibits the HDC activity without any effect on enzymes that inactivate histamine. Therefore, investigating HDC inhibitors similar to (++)-catechin seems to be of great importance.

This paper describes a sensitive, simple, and economical HPLC method for measuring histamine enzymatically produced in vitro by using colorimetric labelling by DABITC of the amino group of histamine. Further, inhibitory effects of three shoyuflavones newly found in...
soy sauce on HDC activities using this method are described.

Materials and Methods

Materials. Isolation and purification of shoyuflavones from soy sauce were done by the procedure described previously. 10 Daidzein and genistein were purchased from Funakoshi Co., Ltd. The HDC from *Clostridium perfringens*, (+)-catechin, L-histidyl-L-phenylalanine (HIS-PHE), 1-methylhistamine, and pyridoxal 5'-phosphate were purchased from Sigma Chem. Co. L-Histidine, diithiothreitol, and polyethylene glycol #300 were purchased from Wako Pure Chemical Industries, Ltd. DABITC was purchased from Dojin Chemical. The partially purified HDC preparation was obtained from a homogenate of mouse mastocytoma P-815 cells by the method of Ohmori et al. 12

Preparation of histamine derivative of DABITC (HA-DABITC) as the authentic compound. HA-DABITC was synthesized by the method of Chang and Creaser. 8 Ten ml of water containing 0.3 g of histamine was mixed with 2 ml of 1 m sodium bicarbonate solution and 20 ml of acetone containing 1.5 g of DABITC. The resulting mixture was refluxed at 90°C for 10 min and then cooled. After evaporating the solution in vacuo, the residue was dissolved in methanol and mixed with 5 g of Celite (Celite Corp.). The methanol was evaporated in vacuo from the mixture and then the residue was subjected to chromatographically separated on a Silica gel 60 (Merck) column (20 mm I.D.×200 mm). After the column was washed with CHCl3, the absorbent reactants were eluted with CHCl3-methanol by increasing the methanol ratio in a stepwise manner. HA-DABITC fractionation checked by TLC were pooled. The elute containing HA-DABITC was collected and evaporated to dryness in vacuo. Recrystallization from acetone gave 0.92 g of pure HA-DABITC as red needles with a melting point of 175-178°C. The purity of HA-DABITC was confirmed by the 1H- and 13C-NMR spectra (in CDCl3), IR spectrum (in KBr), and mass spectrum. The HA-DABITC was very stable for over five weeks (data not shown) at room temperature. L-Methylhistidine derivative of DABITC (HA-Me-DABITC) as the internal standard for HPLC analysis was synthesized by the same procedure.

Assay of the HDC inhibitory activity. The inhibitory activity of isofoxavones against the HDC from P-815 cells was assayed by the method of Ohmori et al. 10 Sixty-five µl of 100 mm phosphate buffer at pH 6.8 containing 0.2 µmol of diithiothreitol, 10 mmol of pyridoxal 5'-phosphate, and 1 mg/ml of polyethylene glycol #300 was mixed with 35 µl (2.2 µU) of the HDC from P-815 cells and 10 µl of isofoxavone dissolved in methanol. The mixture was incubated for 10 min at 37°C. The enzyme reaction was started with adding 10 µl of L-histidine solution (1 mg/ml). After incubation for 14 hr at 37°C, the reaction was stopped by adding 100 µl of acetone.

The inhibitory activity of isofoxavones against the HDC from *Clostridium perfringens* was assayed using L-histidine as a substrate. Ninety µl of Krebs-Ringer bicarbonate buffer, which was adjusted at pH 4.5 with hydrochloric acid, containing 2.7 m NaCl, 10 mmol pyridoxal 5'-phosphate, 2.8 mU HDC, and an appropriate amount of isofoxavone dissolved in dimethylsulfoxide or Krebs-Ringer bicarbonate buffer was incubated for 10 min at 37°C. The reaction was started by adding 10 µl of histidine solution (1 mg/ml). After incubating for 15 min at 37°C, the reaction was stopped by adding 100 µl of acetone.

Derivation of HA-DABITC. After incubation, the histamine produced in each sample was derivatized into the colorimetric compound by treating it with DABITC. Each assay solution prepared as above was mixed with 20 µl of 1 m sodium bicarbonate aqueous solution and 100 µl of 4 m DABITC acetone solution in 1.5 ml of micro tube. The mixture was shaken for 10 seconds by hand and then heated for 5 min in boiling water. After cooling, the reaction mixture was vigorously shaken with 1.0 ml of n-hexane and then n-hexane layer was removed with a Pasteur pipet. Then, 1.0 ml of 1 m NaOH solution and 100 µl of CHCl3 were added to the residual solution. After mixing for 1 min, the mixture was centrifuged for 5 min at 1500 × g. Ten µl of CHCl3 layer diluted with 20-fold CHCl3 was injected into HPLC to measure the HA-DABITC.

HPLC analysis. A Waters 600E multisolvent delivery system with a Waters U6K injector was used for HPLC analysis. The system was operated at room temperature. HPLC analyses were done on a Cosmosil 5S column (4.6 mm I.D. × 150 mm, Nakarai Chem. Co.) with a solvent system of a mixture of CHCl3/N, N-dimethylformamide/H2O (210:90:4) containing 0.4% acetic acid. The flow rate was at 0.8 ml/min, and monitored at 423 nm.

Results

Measurement of histamine as a derivative of DABITC

The reaction temperature significantly influenced reaction rates of HA-DABITC, as shown in Fig. 2. The reaction yields of HA-DABITC at 30, 50, 70, and 100°C were approximately 40, 70, 100, and 100%, respectively. At above 70°C, the reaction rate increased up to 5 min, resulting in the quantitative formation of HA-DABITC. From these data, the optimum reaction conditions for histamine with DABITC in the presence of sodium bicarbonate was at 90°C for 5 min.

Typical HPLC patterns of HA-DABITC analysis are shown in Fig. 3(a–c). HA-DABITC was detected as a single peak at 6.2 min (b). No peak other than the solvent and reagent peaks were detected after incubation of histidine with the HDC from *Clostridium perfringens* (c). When the reproducibility of this method was examined by eight repeated injections of 50 ng of the standard, the coefficients of variation (CV = (standard deviation/mean) × 100) calculated for peak area was 3.00%. The linearity was tested by injecting HA-DABITC in amounts from 5 pg to 500 µg, by adding 10 ng of the internal standard, HA-Me-DABITC; the correlation

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coefficient of the linear calibration curve calculated was 0.997.

**HDC inhibitory activity of isoflavones**

Figure 4(a) shows the effects of isoflavones on the activity of HDC from P-815 cells. The IC$_{50}$ values of shoyuflavones A, B, and C were 1.24, 0.35, and 0.23 mM, respectively (Table 1). Figure 4(b) shows the inhibitory effects of isoflavones on the activity of HDC from *Clostridium perfringens*. The three shoyuflavones
showed the inhibitory activity even in presence of 2.7 m NaCl. The IC$_{50}$ values of shoyuflavones A, B, and C were 1.75, 2.07, and 1.87 mm, respectively (Table 1).

**Discussion**

The described HPLC method is based on a simple, sensitive and specific precolumn derivatization of histamine. Although several sensitive HPLC methods have been developed for histamine analysis, prompt sample treatments are required because of the instability of reagents such as OPA or dansyl chloride and their histamine derivatives as well. Further, some of them need expensive equipment such as a post column. Both DABITC and its derivative of histamine were stable for over five weeks (data not shown) even at room temperature. From these results, this HPLC method was found to be suitable for measuring histamine at the pg level. Therefore, this new method could be effectively applied to measuring the HDC inhibitory activity of isoflavones.

In the study of the effects of shoyuflavones on the activity of HDC from P-815 cells, shoyuflavones B and C were more potent inhibitors than (+)-catechin, and more than their own aglycones, i.e., daidzein and genistein. From these results, the number of hydroxy groups on the A ring and the presence of tartaric acid in position 7 on the isoflavone seems to affect their inhibitory ability against HDC from P-815 cells. Shoyuflavones A and B also strongly inhibited the increase of histamine content without cell toxicity after treatment with 12-0-tetradecanoylphorbol-13-acetate and dexamethasone of the mouse mastocytoma P-815 cell culture (data not shown).

The activity of HDC from *Clostridium perfringens* was more resistant to the inhibitory action of shoyuflavones than that from P-815 cells and was not inhibited by (+)-catechin. Shoyuflavones A and B were more potent inhibitors than their aglycones, such as genistein and daidzein. However, inhibitory activities of isoflavone aglycones at their higher concentrations could not be examined because of their insolubility in the aqueous buffer system. Inhibitory activities of isoflavones seem to be related to the presence of tartaric acid in position 7 but not to the number of hydroxy groups on the A-ring because their inhibitory potential was similar to one another and neither of their aglycones inhibited the HDC activity.

HDCs are found widely not only in animals but in microorganisms. However, structures and features of such HDCs’ differ greatly; such as whether their activities require pyridoxal 5'-phosphate or not. In this research, (+)-catechin inhibited the activity of HDC from P-815 cells but not that of the HDC from *Clostridium perfringens*. Although the three shoyuflavones commonly inhibited activities of HDCs from two sources, their inhibitory activities against two HDCs were slightly different. These results indicated us that activity mechanisms of HDCs between these two sources are different. Histamine produced by HDC is one of representative mediators for gastric acid secretion, and allergic and immunologic reactions. Therefore, the inhibition of HDC activity by shoyuflavones may reduce excess gastric acid secretion and inflammation and result in preventing allergic reactions.

Amounts of shoyuflavones A, B and C in fermented soy sauce ranged approximately from 0.001 to 0.08 mm, which is lower than their IC$_{50}$ values. However, it seems useful to investigate their effects on histamine formation in vivo as well as (+)-catechin because of the lack of acute toxicity in mouse. They may also be useful for clinical use against inflammation, allergy reactions, and excessive gastric acid secretion where histamine is recognized as one of the major causes.

**Acknowledgments**

We wish to thank Professor A. Ichikawa of Kyoto University, Dr. H. Sekine of Noda Institute of Scientific Research, and Dr. T. Aishima and Dr. Y. Ozawa of Kikkoman Corporation for useful and kind advice.

**References**