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Short communication

Method for determination of histidine in tissues by isocratic high-performance liquid chromatography and its application to the measurement of histidinol dehydrogenase activity in six cattle organs

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Abstract

A selective and simple HPLC procedure has been developed to determine histidine (His) and histidinol (HDL) in liver supernate. The separation was performed on a column, Mightysil RP-18 GP. The eluted analytes were measured with UV detection without derivatization which provided detection limits of 1.1 and 2.0 μM for His and HDL (S/N ratio, 3:1), respectively. Recovery of the analytes added to liver sample was 98.3–101.6% within a 1-day study and 95.7–98.6% on different day (6 days) studies. The apparent histidinol dehydrogenase activities (nmol/g wet tissue) at pH 8, 9, 10, 11, and 12 were 38.6, 50.4, 160.3, 274.3, and 185.6 for liver; 90.6, 132.2, 30.7, 22.1, and 6.76 for kidney; 0.0, 0.0, 38.2, 20.1, and 12.9 for pancreas; 0.0, 0.0, 0.0, 14.7, and 6.8 for spleen; 0.0, 0.0, 4.2, 6.8, and 0.0 for muscle; and 0.0, 0.0, 4.9, 1.8, and 0.0 for small intestine, respectively. On the basis of optimum pH values, histidinol dehydrogenase activity in the organs was in the following order: liver>kidney>pancreas>spleen>muscle>small intestine.

Keywords: Histidine; Histidinol dehydrogenase

1. Introduction

With regard to histidine (His) biosynthesis in several prokaryotic and eukaryotic microorganisms such as *Arthrobacter histidinolovorans* [1], *Neurospora crassa* [2], yeast [3], *Bacillus* spp. [4], *Salmonella typhimurium* and *Escherichia coli* K12 [5], and *Escherichia coli* B [6], it has been reported that histidinol dehydrogenase (HLDase) (EC1.1.1.23), is involved in the terminal step of the His biosynthetic pathway. The enzyme catalyzes the two sequential nicotinamide adenine dinucleotide (NAD) linked reactions, leading from histidinol (HDL) via histidinal to the end product His [7]:

$$\text{L-Histidinol} + \text{NAD}^+ \rightarrow \text{L-Histidinal} + \text{NADH} + \text{H}^+$$

$$\text{L-Histidinal} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{L-Histidine} + \text{NADH} + \text{H}^+ \quad (2)$$

The occurrence of HLDase in crude extracts of

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different plant species has been reported [8,9]. This proved that the final step of the His biosynthetic pathway in higher plants is catalyzed by HLDase as in microorganisms.

However, it has not been studied well whether organs of higher animals have HLDase activity as reported in microorganisms and plants. So far, only one attempt was made by the present authors [10]. In order to clarify this point in detail, a convenient determination method for His is needed.

His in the presence of other amino acids has been analyzed by reversed-phase high-performance liquid chromatography (HPLC) after pre-column derivatization [11–13]. However, derivatization is more complex than direct injection. An analytical method based on direct injection generally saves time and is more accurate than a method based on derivative or extraction formation. Recently, a HPLC method (based on direct injection) for the analysis of His and its related compounds has been established [14]. During the anabolic study of His by animal tissues with this method [10], however, it was recognized that a broad peak of NAD appeared just before the peak of His. It is known that NAD must be included in the reaction medium as an essential component as outlined above. However, after one or two sets of sample analyses by the method of Wadud et al. [14], the peak of NAD tended to partially mask the peak of His. In this case, HPLC column replacement was necessary. Alternatively, HPLC column (in the case of used one) regeneration in prescribed form was effective but only for a short time. Therefore, these processes were not economical and also time consuming.

In response to the need to eliminate the above mentioned problems of our previous report [14], we have developed a method based on direct injection for His in the presence of NAD by HPLC with UV detection. As an application of the method, we have determined the activity of HLDase in the incubation of crude extracts of six cattle organs with HDL.

2. Experimental

2.1. Apparatus

A liquid chromatography pump (PU-980), a variable-wavelength ultraviolet detector (UV-970), a three-line degasser (DG-980-50), and a ternary gradient unit (LG-980-02) were obtained from Jasco (Tokyo, Japan); a data analyzer (Chromatopac, C-R6A) and a spectrophotometer (BioSpec-1600) with a thermal printer (Type II, DPU-411) were from Shimadzu (Kyoto, Japan); and a column oven was obtained from Hitachi (Tokyo, Japan); an autosampler (AS-8020) was obtained from Tosoh (Tokyo, Japan) and the Mightysil RP-18 GP column (150× 4.0 mm I.D., 5 μm particle size) was obtained from Kanto (Tokyo, Japan). A homogenizer (Ace Homogenizer, Model AM-7) was purchased from Nihonseiki Kaisha (Tokyo, Japan).

2.2. Reagents

Acetonitrile (HPLC grade) was obtained from Kanto; ultra pure water supplied by a Milli-Q purification system (Nihon Millipore, Tokyo, Japan); NAD and sodium phosphate (monobasic and dibasic) were obtained from Nacalai Tesque (Kyoto, Japan). Metaphosphoric acid (MPA) and phosphoric acid (PA) were obtained from Wako (Osaka, Japan). Histidine was obtained from Kanto and histidinol was purchased from Sigma (St. Louis, MO, USA). 1-Octanesulfonic acid sodium salt was from TCI (Tokyo, Japan).

2.3. Preparation of standard solutions

The standard solutions were prepared by dissolving known amounts of His and HDL containing NAD (4 m*M*) in water and mixed with an equal volume of 3.75% (w/v) metaphosphoric acid to obtain 0 to 1000 μ*M* concentrations. The solutions obtained were filtered through a 0.45-μm membrane filter before HPLC analysis.

2.4. Biological sample preparation and incubation

As a representative of the biological sample, cattle liver was used for the determination of His and HDL. After collection of cattle liver (Japanese Black Cattle) from a local slaughter house (Yoshimoto Meat Industry, Miyazaki, Japan) was quickly placed in ice-cold 0.15 *M* KCl. The tissues were excised, blotted, weighed, and homogenized with 1.5 volumes of 0.15 *M* KCl at 13 000 rpm for 3.0 min below 4 °C. The homogenate was filtered through three

layers of surgical gauze and then centrifuged at 27 000 *g* for 30 min at 4 °C. The supernatant fluid was used as biological sample and also for incubation. A 1-ml portion of the sample containing NAD (4 m*M*) was collected in an Eppendorf tube and mixed with 1 ml of 3.75% (w/v) MPA, refrigerated at 4 °C for a few hours, and then centrifuged at 27 000 *g* for 30 min. The supernatant fluid was filtered through a membrane filter (0.45 μm) and kept at −20 °C until used for HPLC analysis.

In addition to liver, kidney (cortex), pancreas, spleen, and small intestine of cattle (Japanese Black Cattle), cut into small pieces, after freeing from blood, were homogenized in 1.5 ml of 0.15 *M* KCl solution (ice-cold) for each gram of tissue at 13 000 rpm for 3.0 min (exceptions: pancreas 2 min and small intestine 1.5 min) at 4 °C. In the case of muscle, 2.0 ml of 0.15 *M* KCl solution for each gram was used and homogenized at 13 000 rpm for 1.5 min. The homogenates were filtered through two/three layers of surgical gauze and then centrifuged at 27 000 *g* for 30 min at 4 °C. The supernates were used for the assay of HLDase activity. A 4.0-ml portion of the supernate was added to 4.0 ml of Gly–NaOH buffer solution containing $MnCl_2$, NAD, and histidinol (substrate) in a 30-ml Erlenmeyer flask. The final concentrations of Gly–NaOH buffer, $MnCl_2$, NAD, and histidinol were 75, 0.5, 2, and 5 m*M*, respectively. Incubation was carried out at 39 °C for 30 min. After incubation for 0 and 30 min, a 1.0-ml portion of the incubation mixture was taken out from the flask, mixed with 1.0 ml of deproteinizing agent (MPA) in an Eppendorf tube and held for 2 h at 4 °C and centrifuged at 27 000 *g* for 30 min. Then the supernatant fluid was collected for HPLC analysis. Incubation as a control was always run without addition of histidinol.

In all experiments, incubations were carried out in triplicate. The following formula was used to calculate the net production of the product from the substrate:

$$\text{Net production of } X_i = (S_i - S_0) - (C_i - C_0)$$

where X = net amount of product, S = amount of product in the incubation of medium with substrate added, C = amount of product in the incubation of medium with substrate omitted, subscript i = incubation period at 30 min, subscript 0 = incubation period at 0 min.

2.5. Chromatography

Chromatographic separation was performed by isocratic elution. The mobile phase used for separation of His consisted of eluent A_1 [25 m*M* sodium phosphate buffer (pH 2.55, obtained by adding 25 m*M* phosphoric acid) containing 10 m*M* sodium octanesulfonate]–eluent B [acetonitrile–water (90:10)] (89:11). HDL eluted with a separate mobile phase consisting of A_2 [25 m*M* sodium phosphate (monobasic and dibasic) buffer (pH 6.65) containing 10 m*M* sodium octanesulfonate]–[acetonitrile–water (90:10)] (92:8).

Before use, the mobile phase was filtered (membrane filter HV 0.45 μm). The column temperature was maintained at 39 °C in a column oven and all compounds were monitored at 220 nm with UV absorbance detection. The flow-rate was 1.2 ml/min. A 10-μl portion of sample was injected.

2.6. Histidinol dehydrogenase activity

Enzyme (HLDase) activity was expressed in terms of nmol of histidine formed per g tissue per 30 min.

3. Results and discussion

In this study, the absorbance spectra of His and HDL were examined with a spectrophotometer and a wavelength of 220 nm was chosen for the subsequent experiments.

By using a mobile phase consisting of sodium phosphate buffer (solvent A_1)–acetonitrile (solvent B) (89:11, v/v), the peak of His appeared within 17 min (Fig. 1A). Under the chosen conditions, the peak of NAD also did not appear within the time course of His in the chromatogram (Fig. 1A) as observed in our previous method [14]. After elution of His (about 18 min), the proportion of acetonitrile (solvent B) was increased to 95% over 1 min and held for 10 min to wash off the remaining unknown compounds (if any in biological samples). At 30 min after injection, the mobile phase composition was restored to the initial value, and after 15 min the HPLC system was ready for the next run.

Although our primary objective was to separate His from the possible interfering compounds including NAD, we also tried to quantify HDL by chroma-

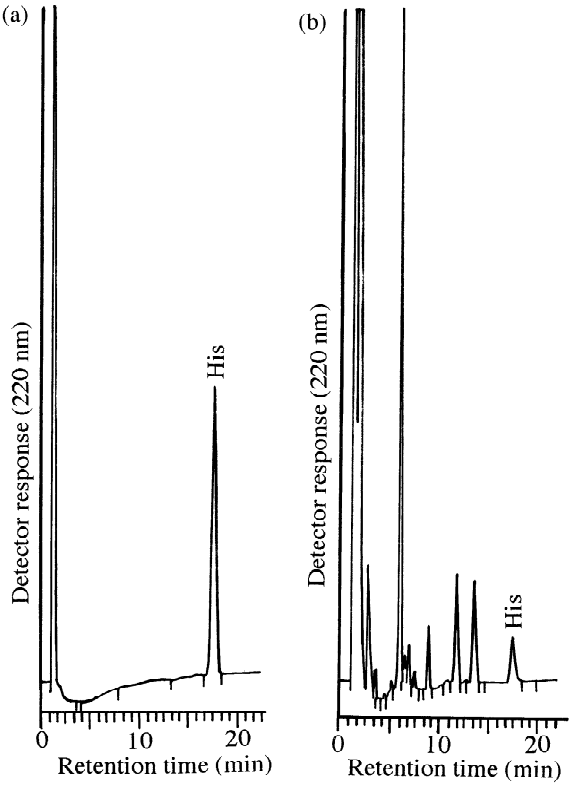

Fig. 1. (A) Chromatogram of standard His (0.25 m*M*) by HPLC. His = Histidine. (B) Typical chromatogram of deproteinized liver sample by HPLC. His = Histidine.

tography. However, attempts to elute HDL with His in one chromatogram failed. Therefore, HDL was eluted separately in another chromatogram. In this case, HDL was eluted at 20 min when sodium phosphate buffer (solvent A_2)–acetonitrile (solvent B) (92:8) was used (figure not shown). After elution of HDL, the percentage of acetonitrile was increased, i.e., from 21 min, to buffer–acetonitrile (17:83) and held for 10 min. At 32 min after injection, the mobile phase composition was restored to the initial value and held for another 15 min for column equilibration.

The calibration curve of peak height versus standard solution concentration was linear over the range 3.7–1000 μ*M* for His and 6.7–1000 μ*M* for HDL with $r = 0.999$ and 0.999, respectively. Linear regression equations are provided in Table 1. The limits of detection (LODs) of the compounds as estimated by injecting successively lower concentrations until a signal-to-noise ratio of approximately 3 was obtained, are provided in Table 1. The limits of quantification (LOQs) of the compounds at a signal-to-noise (S/N) ratio of 10:1 is also presented in Table 1.

The purity of the compound in each peak was confirmed by comparing peak height ratios of the authentic compounds in standard solutions and of the identified peaks in liver samples at different wavelengths (220, 228 nm), according to Lefeng et al. [15]. The ratios of absorbance of the peaks at the two wavelengths for the liver samples were in good agreement with those found for the respective pure compounds (Table 1). This means that at the selected wavelengths there were no significant contributions to the peak heights from compounds in the liver samples other than the selected analytes. Method precision was measured by the relative standard deviation (RSD). At different concentrations of the standard samples added to the liver samples, the within-day RSD varied between 0.44 and 3.23% and the between-day (total 6 days) RSD was 0.73–3.88%. Recoveries of His and HDL added to liver samples ranged from 98.3 to 101.6% for the within-day study and 95.7–98.6% on different day (6 days) studies. A detectable amount of His was present in the supernate containing NAD (80.4 nmol/g wet

Table 1
Limit of detection (LOD), limit of quantification (LOQ), linear regression equation and peak height ratio at 220 and 228 nm of His and HDL[a]

Compound	LOD (μ*M*)	LOQ (μ*M*)	Linear regression equation	Peak height ratio (220/228)	
				Standard sample	Liver sample
Histidine (His)	1.1	3.68	$y = 235.3 + 50.028x$	3.531	3.498
Histidinol (HDL)	2.0	6.72	$y = 131.36 + 21.759x$	3.482	3.436

[a] y = Peak height (μV), x = concentration (μ*M*).

liver) (Fig. 1B) while HDL was not detected (figure not shown).

3.1. Application of the method

In order to examine the usefulness of this method, we applied it to the measurement of the activity of HLDase in crude extracts prepared from six different organs of cattle as shown in Fig. 2. The apparent HLDase activities (nmol/g tissue) at pH 8, 9, 10, 11, and 12 were 38.6, 50.4, 160.3, 274.3, and 185.6 for liver; 90.6, 132.2, 30.7, 22.1, and 6.76 for kidney; 0.0, 0.0, 38.2, 20.1, and 12.9 for pancreas; 0.0, 0.0, 0.0, 14.7, and 6.8 for spleen; 0.0, 0.0, 4.2, 6.8, and 0.0 for muscle; and 0.0, 0.0, 4.9, 1.8, and 0.0 for small intestine, respectively (Fig. 2). The present results demonstrate that all these organs have HLDase activity and are capable of biosynthesizing His to various extents. In this regard, we reported the HLDase activity of cattle liver and kidney elsewhere [10]. The present result (when incubation temperature was set at 37 °C) (data not shown) for liver and kidney was almost similar with the previous one (incubation temperature set at 37 °C), which also indicate the reproducibility, reliability, and accuracy of our proposed method. However, present data (at 39 °C) shown in Fig. 2 were slightly higher compared with previously published data [10], which seemed due to elevated incubation temperature (i.e., at 39 °C) employed in the present experiments, and

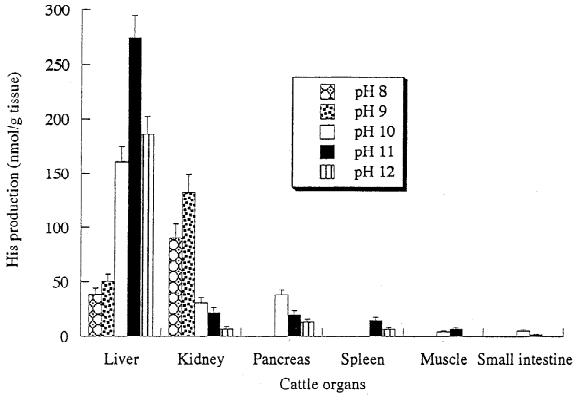

Fig. 2. Effect of pH on the histidinol dehydrogenase activities of crude enzyme solutions of cattle liver, kidney, pancreas, spleen, muscle, and small intestine at 39 °C in 30-min incubation.

maximum activity was at 39 °C tested at the range of 27 to 72 °C (data not shown).

The optimum pH values of liver, spleen and muscle were observed at 11.0 with maximum activity, while those of pancreas and small intestine showed maximum activities at 10.0 and that of kidney at 9.0. This probably reflects the differences in optimum activity from one organ to another. However, on the basis of optimum pH values, HLDase activity in the unit weight of organs was in the following order: liver>kidney>pancreas>spleen>muscle>small intestine. In fact, there was no evidence of any activity of HLDase in the pancreas, spleen, muscle, and small intestine so far. Thus, it appeared for the first time that histidinol dehydrogenase, the enzyme responsible for the conversion of histidinol to His, exists in animal organs such as pancreas, spleen, muscle and small intestine; although enzyme activities were much lower compared with liver and kidney.

4. Conclusions

A selective, sensitive, reproducible, and simple HPLC method for determination of His in liver sample, based on isocratic elution, has been developed. Neither specialized sample preparation nor chemical derivatization is required for analysis of this compound. With this method, it has been revealed that liver, kidney, pancreas, spleen, muscle, and small intestine have histidinol dehydrogenase activities.

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References

[1] M.R. Dhawale, E.H. Creaser, J.C. Loper, J. Gen. Microbiol. 73 (1972) 353.
[2] A.C. Minson, E.H. Creaser, Biochem. J. 114 (1969) 49.
[3] J.K. Kessey, R. Bigelis, G.R. Fink, J. Biol. Chem. 254 (1979) 7427.
[4] J.A. Lindsay, E.H. Creaser, Biochem. J. 165 (1977) 247.
[5] K.G. Bitar, J.R. Firca, J.C. Loper, Biochim. Biophys. Acta 493 (1977) 429.
[6] N. Andorn, J. Aronovitch, J. Gen. Microbiol. 128 (1982) 579.
[7] E. Adams, J. Biol. Chem. 209 (1954) 829.
[8] A. Nagai, A. Scheidegger, Arch. Biochem. Biophys. 284 (1991) 127.
[9] Y.-S. Wong, M. Mazelis, Phytochemistry 20 (1981) 1831.
[10] S. Wadud, R. Onodera, M.M. Or-Rashid, Anim. Sci. J. 72 (2001) 253.
[11] D.C. Turnell, J.D.H. Cooper, Clin. Chem. 28 (1982) 527.
[12] R.A. Bank, E.J. Jansen, B. Beekman, J.M. te Koppele, Anal. Biochem. 240 (1996) 167.
[13] P. Fürst, L. Pollack, T.A. Graser, H. Godel, P. Stehle, J. Chromatogr. 499 (1990) 557.
[14] S. Wadud, R. Onodera, M.M. Or-Rashid, J. AOAC Int. 83 (2000) 8.
[15] Z. Lefeng, Y. Yunlu, Y. Ruiyu, J. Chromatogr. 282 (1983) 333.