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Notes

Determination of Hydrogen Peroxide by High-Performance Liquid Chromatography with a Cation-Exchange Resin Gel Column and Electrochemical Detector

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Hydrogen peroxide (H_2O_2) forms in food and living organisms[1,2] and has been utilized as a sanitizer and a disinfectant in the food industry.[3] H_2O_2 mediates various cellular injuries including mutagenesis and carcinogenesis. For example, oral administration of H_2O_2 has been found to induce duodenal tumors in mice.[4] Thus, an analysis of H_2O_2 in food is very important.

Conventional methods for the analysis of H_2O_2 in food and biological materials are usually based on its redox reaction.[5-9] It is necessary to eliminate redox substances such as ascorbic acid and polyphenols from the sample prior to each analysis by these methods, because these substances interfere with the reaction in H_2O_2 determination.[10] Direct H_2O_2 measurement by high-performance liquid chromatography (HPLC) would not require such sample pretreatment. There are, however, only a few reports on H_2O_2 separation and H_2O_2 detection by HPLC. Miyazawa *et al*. reported luminol chemiluminescent determination of H_2O_2 by HPLC with a cation-exchange resin gel column (a Shodex Ionpak KS-801).[7] They reported that a column with distilled water as the mobile phase allowed good separation, without the irreversible binding of H_2O_2 to the column surface which usually occurs in other columns. Although their method for H_2O_2 measurement is selective and sensitive, the electrochemical detector (ECD) is more popular than the chemiluminescent detector; the electrolyte for electrochemical reaction might also be less expensive than microperoxidase, which is used for the postcolumn reaction for the luminol chemiluminescent determination. ECD has been used for H_2O_2 detection by HPLC to measure certain compounds such as acetylcholine.[11] Acetylcholine separated with an octadecylsilyl (ODS) column reacts with acetylcholinesterase and choline oxidase to form betaine and H_2O_2. Then the H_2O_2 is measured by ECD. The mobile phase including electrolytes was used throughout, *i.e*. separation, postcolumn reaction, and detection, because acetylcholine instead of H_2O_2 was separated.

We report a method for separating H_2O_2 by HPLC with ECD. One problem in using the Shodex Ionpak KS-801 as a cation-exchange resin gel column and pure water as the eluent is the absence of an electrolyte for electrochemical detection. Therefore, the eluent of the column was mixed with an electrolyte prior to electrochemical detection. Under adequate conditions, 2 pmol H_2O_2 was detected, which is comparable to the results of the luminol chemiluminescent determination. H_2O_2 amounts in various samples containing various redox substances such as coffee and ascorbic acid solutions were also measured by this method.

Experimental

Reagents

Sodium sulfate (Na_2SO_4), ethylenediamine-*N,N,N′,N′*-tetraacetic acid disodium salt (EDTA·2Na) and ascorbic acid were obtained from Wako (Osaka, Japan). Hydrogen peroxide (31% aqueous solution) was purchased from Mitsubishi Gas Chemical (Tokyo, Japan).

HPLC

A schematic diagram of the HPLC system for H_2O_2 determination is shown in Fig. 1a. The system consisted of an HPLC column packed with a cation-exchange resin gel of sulfonated styrene-divinylbenzene copolymer (Shodex Ionpak KS-801, 300 mm×8 mm i.d.; Showa Denko, Tokyo, Japan), two HPLC pumps (Jasco PU-980; Japan Spectroscopic, Tokyo, Japan) with a Rheodyne Model 7125 sample loop injector (20 μl or 100 μl) and an ECD (Jasco 840-EC).

All aqueous solutions were prepared with distilled water treated with a Milli-Q Labo system (Millipore, Bedford, MA, USA). The electrolyte was prepared

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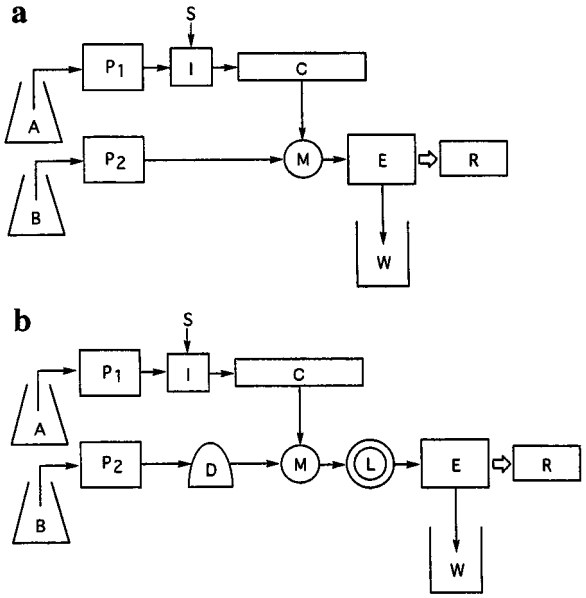

Fig. 1 Schematic diagram of the HPLC system for hydrogen peroxide determination. a: A, mobile phase (pure water); B, electrolyte (10, 50 or 250 mM Na_2SO_4); P_1 and P_2, pumps; I, sample injection valve; S, sample (20 μl or 100 μl); C, HPLC column; M, mixing unit; E, electrochemical detector; R, recorder and integrator; W, waste. b: D, dumper; L, loop (10 m); other abbreviations are the same as in the legend of Fig. 1a.

with Na_2SO_4 (10, 50 or 250 mM) and EDTA·2Na (10 μM). The electrolyte and pure water were filtered through a cellulose nitrate filter (pore size 0.45 μm; Advantec Toyo, Tokyo, Japan) prior to use. The flow-rate of pure water from the first pump through a KS-801 column was 0.60 ml/min and that of the electrolyte from the second pump was 0.15 ml/min. ECD was equipped with a Pt electrode as a working electrode and an Ag/AgCl electrode as a reference electrode. The peak areas were calculated with a Chromatocorder 21J (System Instruments, Tokyo, Japan).

Results and Discussion

Optimum conditions of HPLC for H_2O_2 determination

Figure 2 shows a typical chromatogram of a standard solution of H_2O_2 (2 nmol). H_2O_2 was detected with ECD, whose applied potential was set at 500 mV *vs.* Ag/AgCl, in the presence of an electrolyte (10 mM Na_2SO_4). A peak ascribed to H_2O_2 appeared at 21.2 min. A peak at 16.8 min is considered to be due to an impurity in the authentic H_2O_2 solution. The peak area of H_2O_2 increased with the concentration of electrolyte up to 250 mM (data not shown). Under these conditions, the baseline fluctuated at a constant rate owing to the pulsed flow of the electrolyte from the second pump and the determination of H_2O_2 was disturbed. The signal-to-noise ratio at 2 nmol of H_2O_2 was 8.3. In order to decrease the wave height of the pulse, we used a coiled tube (10 m) as a loop between the mixing unit and the ECD and also a dumper (Shodex DP-1) between the second pump and the mixing unit (Fig. 1b). Consequently, the wave height of the pulse noticeably decreased and the sensitivity to detect H_2O_2 increased. The signal-to-noise ratio at 2 nmol of H_2O_2 was 28.7.

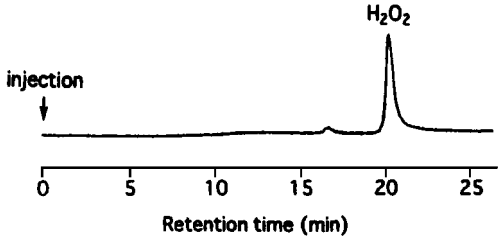

Fig. 2 Typical chromatogram of hydrogen peroxide (2 nmol) separated with an Ionpak KS-801 column. The applied potential of ECD was set at 500 mV *vs.* Ag/AgCl. Other analytical conditions were the same as in the legend of Fig. 1.

Since the higher voltages such as 1 V *vs.* Ag/AgCl resulted in a quick formation of a coating of contaminants on the surface of the platinum electrode, the applied potential was set at 400 mV in the following experiments. With the optimized HPLC-ECD conditions, the calibration line from 2 pmol to 20 nmol of H_2O_2 showed good linearity (correlation coefficient r=0.9984). The relative standard deviation (RSD) for each H_2O_2 concentration did not exceed 5%. The detection limit of the total system was 0.2 pmol of H_2O_2 (signal-to-noise ratio=3). Since the detection limit of H_2O_2 by the HPLC chemiluminescent method was reported to be 4 pmol, our HPLC-ECD method has the higher sensitivity.

Fast analysis of H_2O_2

The retention time of H_2O_2 (21.3 min) might be too long for measurements, when many samples must be analyzed or if H_2O_2 is easily decomposed. In order to analyze H_2O_2 with a short retention time, we employed an Ionpak KS-801S as an HPLC column. The same resin as used in KS-801 was packed in the column, which was half as long (150 mm) as the KS-801 column. Analytical conditions except the flow rates of water (1.00 ml/min) and of the electrolyte (0.25 ml/min) were the same as those with KS-801. Under these conditions, we were able to determine H_2O_2 with a shorter retention time (8.7 min).

Application

We quantified the H_2O_2 concentration in coffee. Coffee was prepared by dissolving 2.0 g of commercial freeze-dried instant coffee granules in 140 ml of boiling water. The coffee solution was diluted 50 times with water, passed over a SepPak C_{18} (Millipore) and filtered through a cellulose nitrate filter disc (Ekicrodisk 13, pore size 0.45 μm, Gelman Science Japan, Tokyo, Japan) to remove particles. Since no peak ascribed to H_2O_2 was found by the treatment of the sample solution with catalase before the filtration procedure, the detect-

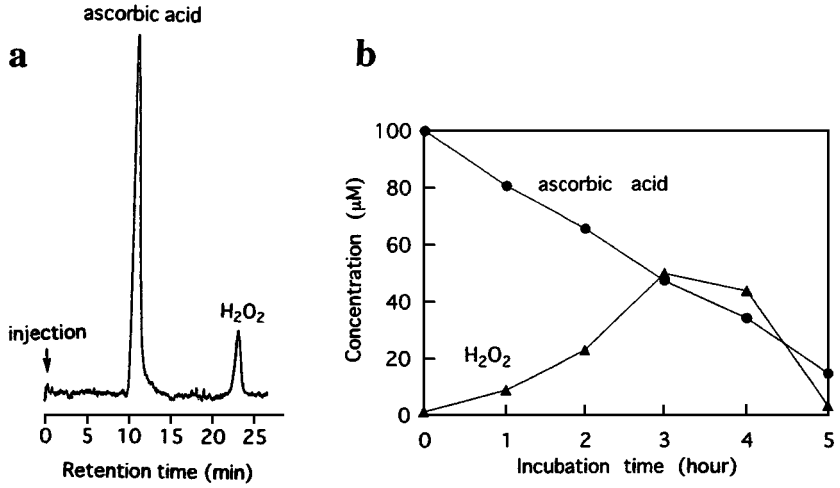

Fig. 3 Analysis of hydrogen peroxide formed during autoxidation of ascorbic acid. a: a typical chromatogram of the ascorbic acid solution (100 μM) incubated for 3 h. b: time dependency of the concentration of ascorbic acid and H_2O_2 in the aqueous solution of ascorbic acid.

ed peak was ascribed to H_2O_2. The H_2O_2 content in a coffee drink was 73.8±4.6 μM (n=4). The value approximated that previously determined by a chemiluminescent method.[7]

The mechanism of H_2O_2 formation during autoxidation of ascorbic acid is not clear, because it is difficult to determine H_2O_2 in the presence of large amounts of ascorbic acid by conventional methods using redox reactions. Thus, autoxidation of ascorbic acid was investigated by our HPLC method, which enabled us to measure H_2O_2 and ascorbic acid simultaneously. An aqueous solution of ascorbic acid (100 μM) was incubated at room temperature. The solution (20 μl) was collected every hour and filtered with a cellulose nitrate filter disc. Figure 3a shows the chromatogram of the sample incubated for 3 h. The peak of ascorbic acid appeared at 10.9 min and that of H_2O_2 at 23.3 min. Figure 3b shows the time dependency of the concentrations of ascorbic acid and H_2O_2. The concentration of ascorbic acid was calculated by fitting the area of ascorbic acid at 0 h as 100 μM. The concentration of ascorbic acid linearly decreased with the standing time, while that of H_2O_2 increased for 3 h and then decreased. These results suggest that the formation of H_2O_2 during autoxidation of ascorbic acid was decreased by the reaction with some degradation products of ascorbic acid such as diketogulonate and 3,4,5-trihydroxy-2-ketopentanoate.[12,13]

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