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ENZYMATIC DETERMINATION OF HYDROGEN PEROXIDE USING GAS CHROMATOGRAPHY

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SUMMARY

A new method for the gas chromatographic determination of hydrogen peroxide using a methanol–catalase system is described. This method involves the enzymatic conversion of hydrogen peroxide to formaldehyde and derivatization of the formaldehyde formed with pentafluorobenzyloxylamine. The factors affecting the reaction in the methanol–catalase system such as the pH of the reaction solution, the reaction period, the catalase and methanol concentrations and the presence of a reducing substance were investigated. A linear calibration graph passing through the origin was obtained in the range 0.06–1.2 μg of hydrogen peroxide in 2.0 ml of aqueous solution for electron-capture detection.

INTRODUCTION

The determination of hydrogen peroxide has been effected by enzymatic colorimetric[1] or fluorimetric[2] methods using a combination of peroxidase and various indicator substrates, which are oxidized by hydrogen peroxide in the presence of peroxidase as a catalyst to yield a chromogen or a fluorophore. This technique has been applied to the sensitive determination of oxidative enzyme activities such as peroxidase[1-3], glucose oxidase[1,3], xanthine oxidase[1,3] and galactose oxidase[4], and to the determination of substrates such as hydrogen peroxide[3], glucose[3,4], galactose[4] and hypoxanthine[3] in these enzyme systems. One of the advantages in using an enzyme as an analytical reagent is the specificity of the reaction. However, the effect of peroxidase, which catalyses oxidation by hydrogen peroxide, is not so specific for the substrate and the oxidation of an indicator substrate by hydrogen peroxide catalysed with peroxidase is easily influenced by the presence of a reducing substance such as ascorbic acid[5].

On the other hand, hydrogen peroxide produces formaldehyde in the presence of a large amount of methanol and catalase[6]. This principle was applied to the colorimetric determination of uric acid in serum and urine with a uricase–catalase system[7], in which the reaction of formaldehyde and acetylacetone was used for colour development. The formaldehyde thus formed can also be measured by gas chromatography (GC) or high-performance liquid chromatography as a suitable derivative.

Pentafluorobenzyloxylamine (PFBOA) has been found to be an excellent derivatizing agent in the GC determination of low-molecular-weight carbonyl compounds in aqueous solution[8,9], and has been applied successfully to the sensitive determination of amygdalin in aqueous solution in which the benzaldehyde liberated from enzymatic hydrolysis of amygdalin was converted by PFBOA into its O-pentafluorobenzyl oxime (O-PFBO) and the derivative was determined by GC[10].

In this paper, a method for the GC determination of hydrogen peroxide using the methanol–catalase system is described. This method is based on the combination of an enzyme reaction and GC.

EXPERIMENTAL

Reagents

PFBOA hydrochloride (melting point 215°C) was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan)[8]. Iodobenzene was used as an internal standard (IS). A 0.3% solution of hydrogen peroxide was prepared by diluting a 30% stock solution (Merck, Rahway, NJ, U.S.A.) with distilled water. The hydrogen peroxide concentration was determined by iodimetry. The solution was stable for 1 month in a refrigerator.

Catalase

Catalase (270,000 U/ml) was obtained from Boehringer (Mannheim, G.F.R.). A stock solution (20,000 U/ml) was prepared by diluting it with distilled water.

Apparatus and conditions

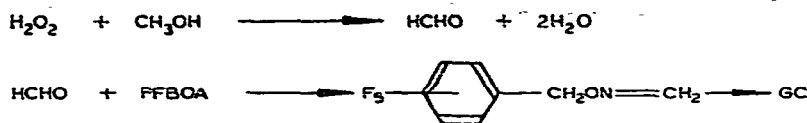A Shimadzu GC-4CPF gas chromatograph equipped with a hydrogen flame-ionization detector (FID) and a GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector (ECD) were used. A 2-m glass column packed with 3% XE-60 on 80–100-mesh Celite 545 AW DMCS was used, with a column temperature of 100°C, a detector temperature of 150°C and a chart speed of 0.25 cm/min.

Standard procedure

To 2.0 ml of sample solution containing hydrogen peroxide in a 10-ml test-tube were added 0.5 ml of methanol, 0.1 ml of catalase solution and 0.5 ml of aqueous PFBOA solution (1 mg/ml). The components were mixed and the tube was placed in a water-bath at 37°C for 30 min, except for the study of the time course. After saturation with sodium chloride and acidification with 1 drop of 18 N sulphuric acid, the O-PFBO derivative of formaldehyde was extracted with 0.3-ml of n-hexane containing iodobenzene (182 μg/ml) was an IS. Excess of sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle. An aliquot of the extract was applied to the GC column. Quantitation was carried out using calibration graphs obtained from known amounts of hydrogen peroxide. Blank tests were performed by omitting hydrogen peroxide.

RESULTS AND DISCUSSION

The principle of the reactions using methanol and catalase is as follows:

$$H_2O_2 \quad + \quad CH_3OH \quad \longrightarrow \quad HCHO \quad + \quad 2H_2O$$

$$HCHO \quad + \quad PFBOA \quad \longrightarrow \quad F_5 \text{—} \langle \bigcirc \rangle \text{—} CH_2ON\!=\!CH_2 \longrightarrow GC$$

A typical GC separation of formaldehyde as its O-PFBO derivative is illustrated in Fig. 1. Iodobenzene was used as an IS. The reaction of formaldehyde with PFBOA proceeded readily at room temperature. The resulting derivative was extractable from the aqueous solution with n-hexane, and was very volatile and extremely sensitive to the ECD. According to the standard procedure described under Experimental, the following factors affecting the reaction in methanol–catalase system were investigated.
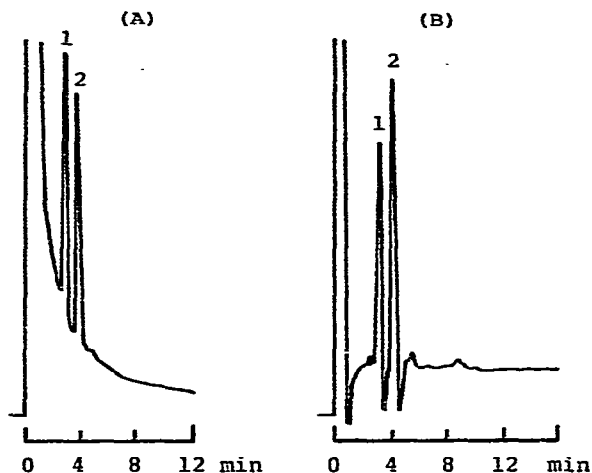
Fig. 1. Gas chromatogram of formaldehyde produced from oxidation of hydrogen peroxide by catalase on a 2.0-m 3% XE-60 column at 100°C. 1 = formaldehyde O-PFBO; 2 = iodobenzene. (A) Analysis on a gas chromatograph equipped with an FID, using 2.0 μg of hydrogen peroxide. (B) Analysis on a gas chromatograph equipped with a ^{63}Ni ECD, using 200 ng of hydrogen peroxide.

pH in the reaction solution

Using 30 μg of hydrogen peroxide, the optimum pH for the catalase reaction was examined in the range 2–8 and the results are shown in Fig. 2. The pH in the reaction solution was adjusted with dilute hydrochloric acid, 0.1 M acetate buffer or 0.1 M phosphate buffer. The measured values of peak-height ratios to the IS were constant at pH 3–8. Considering these results, sample solutions were assayed without using any buffer solution.

Reaction period

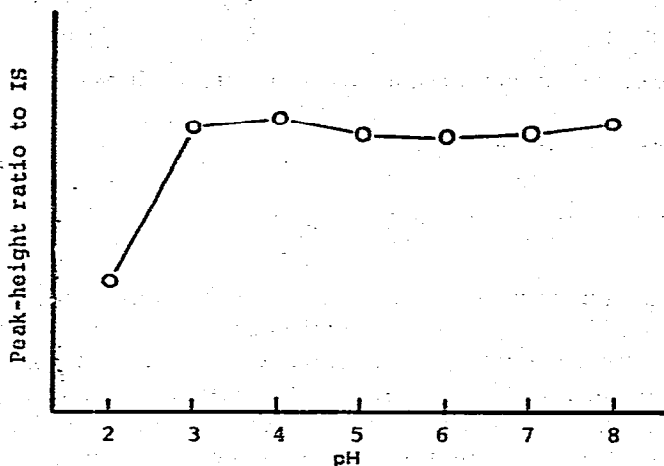Using 30 μg of hydrogen peroxide, the effect of the reaction period was in-

Fig. 2. Effect of pH on the catalase reaction with hydrogen peroxide in the presence of methanol.

vestigated through the entire procedure. The results in Fig. 3 show the overall effects on the catalase reaction and on the condensation reaction of formaldehyde produced with PFBOA. It can be seen that the reaction was satisfactory in 40 min, after which the measured values were constant for up to 120 min.
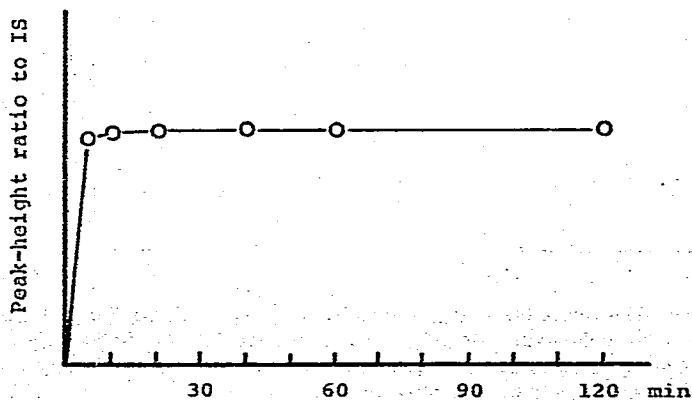

Fig. 3. Effect of reaction period on the method.

Catalase concentration

The necessary concentration of catalase for the determination of hydrogen peroxide was examined by the standard method. As shown in Fig. 4, above 100U of catalase constant values were obtained for 30 μg of hydrogen peroxide.

Methanol concentration

Using 30 μg of hydrogen peroxide, the optimal concentration of methanol for the catalase reaction was examined and was found to be between 8.0 and 30%, as shown in Fig. 5.
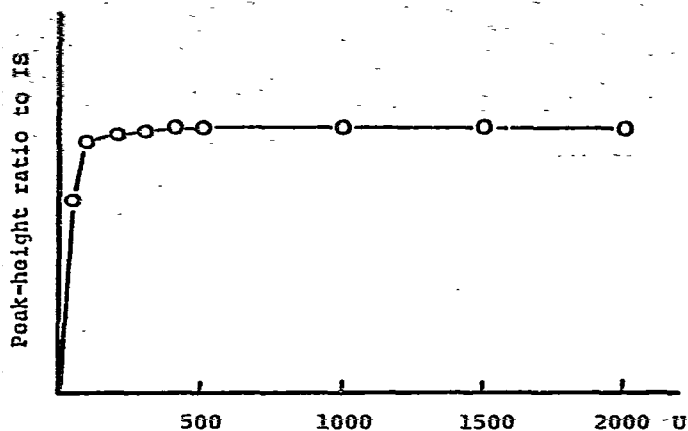
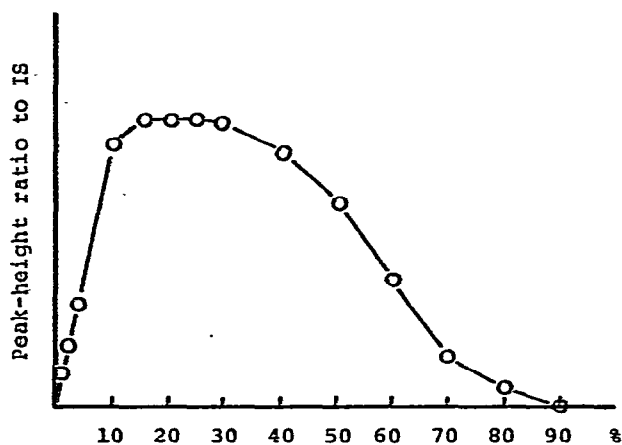Fig. 4. Effect of catalase activity.

Fig. 5. Effect of methanol concentration.

Influence of reducing compounds

As a good example of reducing compounds, the possibility of interference by L-ascorbic acid was examined. L-Ascorbic acid was added to 3 ml of the sample solution at a level of 5–45 μg with 30 μg of hydrogen peroxide. The results are shown in Fig. 6.

The observed levels of hydrogen peroxide decreased slightly as the amount of L-ascorbic acid increased, but the extent was not sufficient to cause practical problems. From the above results, the standard procedure was established.

Application

A sample solution containing an individual amount of hydrogen peroxide was measured according to the standard procedure and calibration graphs were constructed by plotting the ratio of the peak height of formaldehyde to that of the IS, and straight lines passing through the origin were obtained in the range 1.5–60 μg of hydrogen peroxide in 2.0 ml of aqueous solution for the FID and 0.06–1.2 μg for the
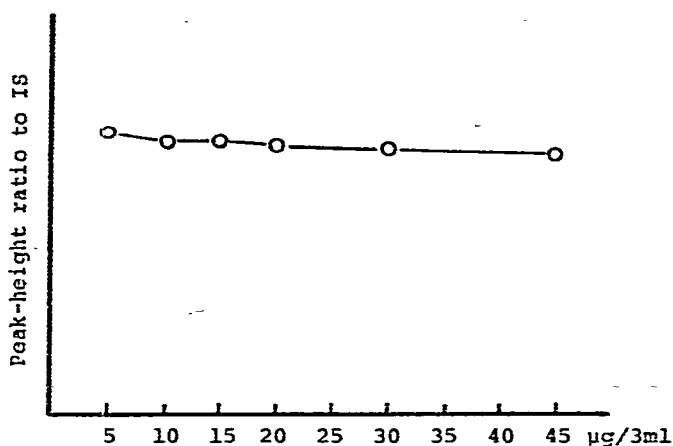
Fig. 6. Effect of presence of ascorbic acid.

ECD. The correlation coefficients for the calibration graphs were 0.9996 for the FID and 0.9995 for the ECD. The reproducibility of the method on an identical sample solution containing 30 μg of hydrogen peroxide in 2 ml of aqueous solution for the FID and 0.3 μg for the ECD was examined. The coefficients of variation obtained were 1.39% ($n = 5$) for the FID and 1.28% ($n = 5$) for the ECD. For the determination of hydrogen peroxide the present method is superior to the peroxidase method as it is not subject to interferences from coexisting reducing compounds, so it has a higher specificity for hydrogen peroxide and gives a reliable value. The O-PFBO derivative of formaldehyde is also sensitive forwards the ECD, but the blank values were found to be high, corresponding to about 0.05 μg of hydrogen peroxide in 2 ml of sample solution, so the detection limit of hydrogen peroxide was about 0.01 μg/ml in sample solutions. Reduction of the blank values will be the subject of a future study.

From the present study, it is concluded that the proposed GC method for the determination of hydrogen peroxide in aqueous solution is satisfactory with respect to specificity, sensitivity and simplicity. The study was performed in order to develop an enzymatic technique for the assay of a variety of oxidative enzymes, substrates which produce hydrogen peroxide by enzyme reactions, and activators and inhibitors of such systems. A study of the GC determination of glucose in serum with the glucose oxidase–catalase system is currently being performed, and will be reported in the near future.

ACKNOWLEDGEMENTS

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