Environ. Sci., Hokkaido	9 (2)	195~206	Dec. 1986

An Enzyme-Linked Immunosorbent Assay (ELISA) for Measuring Human Erythrocytes Catalase

Masaaki Kurasaki[1], Keizo Ito[2], Mika Suzuki-Kurasaki[1], Takeshi Saito[2], Tadao Makizuka[2], Yutaka Kojima[1] and Kazuo Saito[2]

1: Department of Environmental Medicine, Graduate School of Environmental Science, Hokkaido University, Sapporo, Japan
2: Department of Hygiene and Preventive Medicine, School of Medicine Hokkaido University, Sapporo, Japan

Abstract

A sensitive enzyme linked-immunosorbent assay (ELISA) for human erythrocytes catalase has been developed at the first time by using polyclonal (rabbit) anti-human erythrocytes catalase. This assay system consists of immobilization of an antibody on commercial immunoplate and of conjugation of the antibody with alkaline phosphatase. The assay for catalase has high sensitivity and specificity, 5 fmol of catalase in erythrocytes is detectable. In addition, catalate in human erythrocytes either fully or partially inactivated by enzyme inhibitors could be revealed by the ELISA.

Key Words: Enzyme immunoassay, catalase, human, erythrocytes.

Introduction

Catalase (EC 1.11.1.6.) is an enzyme which catalyzes the decomposition of hydrogen peroxide to oxygen and water in the superoxide anion metabolic pathway (Lal et al., 1980). Its biological role in the erythrocyte seems to be the protection of hemoglobin and cell components against oxidizing agents (Geerts and Roels, 1982). Recently, this enzyme has also received attention in the field of practical medicine in relation to superoxide dismutase (SOD) (EC 1.15.1.1.). Catalase and SOD activities in normal healthy adults and patients with cancer and other various diseases have been investigated in our laboratory (Saito et al. 1982; 1984; Kurasaki et al. 1982; 1986; Kaji et al. 1985). Contents of catalase in erythrocytes have been reported by Stansell and Deutsch (1966), Hartz et al. (1973) and Ben-Yoseph and Shapira (1973). Their methods were similar to those of Mancini et al. (1965) which have low sensitivity and required too much time. In this paper, we describe a specific and sensitive ELISA employing the sandwich method developed by the use of anti-catalase-alkaline phosphatase conjugates and polystyrene beads coated with anti-

Correspondence: Masaaki Kurasaki, Department of Environmental Medicine, Graduate School of Environmental Science, Hokkaido University, Nishi-5, Kita-10, Kita-ku, Sapporo 060, Japan.

catalase antibodies prepared from antisera against human erythrocyte catalase.

Materials and Methods

Materials

Human blood was purchased from the Hokkaido Red Cross Blood Centre. Sephadex G-200 and Sepharose CL-4B were obtained from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was obtained from Whatman. ATTO Instruments (Tokyo) were employed for electrophoresis. Ultrafilters were products of Amicon Corp. Nunc authorized immunoplates I were purchased from Nunc. Alkaline phosphatase Lot No. P-2251 and BSA were obtained from Sigma. Freund's complete adjuvant was obtained from Difco. Other chemicals were prepared from Nakarai Chemicals (Osaka).

Methods

Purification of human erythrocyte catalase

Purified catalase from human erythrocytes was obtained by our original method. All purification procedures were carried out at 4°C. Human blood was centrifuged at 800×g for 20 min and the buffy coat and plasma were discarded. The cells were washed three times with cold isotonic sodium chloride, and lysed by the addition of distilled water. The hemolyzed erythrocytes were dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and applied to a column (10 cm×11 cm) containing DE-52 equilibrated with the dialysis buffer. The solution containing the catalase activity was eluted batchwise with 50 mM phosphate buffer, pH 6.85, containing 100 mM NaCl. It was concentrated to 300 ml by ultrafiltration using a PM 10 filter, and dialyzed against 10 mM phosphate buffer, pH 6.85. The dialysed solution was rechromatographed with a DE-52 column (2.4 cm×76 cm) and eluted with a gradient from 10 mM phosphate buffer, pH 6.85, to 50 mM phosphate buffer containing 100 mM NaCl and 100 mM KCl, pH 6.85. Solid ammonium sulphate was added to the fractions containing catalase until its concentration reached 35% of saturation. The mixture was stirred for 120 min and centrifuged at 8000×g for 30 min. Further, solid ammonium sulphate was added to the supernatant solution containing catalase until its concentration reached 60% of saturation. The mixture was stirred overnight and then centrifuged at 8500×g for 40 min. The precipitate containing more than 60% of the catalase activity was resuspended in 100 mM phosphate buffer, pH 7.1. The concentrated catalase solution was applied to a column (2.8 cm×80 cm) of Sephadex G-200 equilibrated with 100 mM phosphate buffer, pH 7.1. The elution pattern was shown in Fig. 1. The major peak fractions with an absorbance at 408 nm were concentrated.

Preparation of antisera of human erythrocytes catalase

For raising antisera in rabbits, the purified catalase was injected subcutaneously as a water-in-oil emulsion in Freund's complete adjuvant, and it was boosted 5 times. The amount of purified catalase used for immunizing the rabbits was 3.3 mg

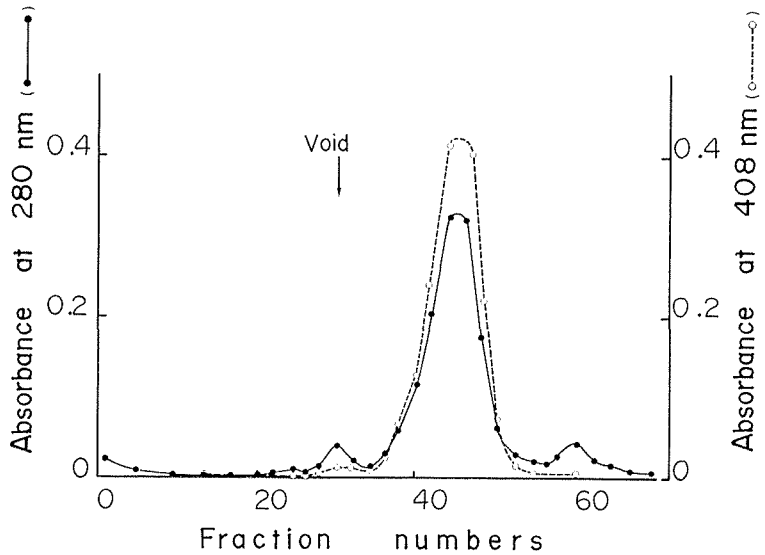

Figure 1. Sephadex G-200 chromatography of human erythrocytes catalase. The ammonium sulphate precipitation was applied to a column (2.8×80 cm) of Sephadex G-200. The enzyme was eluted as described under "Materials and Methods".

per animal. The specificity of the antisera obtained against erythrocyte catalase was checked by the agar-gel double-diffusion test. This antisera was used for the present investigation.

Preparation of an antigen affinity column

A human erythrocyte catalase affinity column was prepared by the method of Cuatrecasas (1970) and, Axen and Ernback (1971) with some modifications.

Sepharose CL-4B was washed three times with 10 volumes of cold distilled deionized water on a glass filter. The gel was resuspended in 30 ml of distilled water. The initial pH of the solution was adjusted to 12.2 with 4 M sodium hydroxide. Ten grams of cyanogen bromide were suspended in the solution containing Sepharose CL-4B gel. A desired pH (11-12) of the solution was maintained with addition of 4 M sodium hydroxide. The suspension was stirred during the reaction and the temperature was kept at 23-27°C. The time of reaction at the fixed pH was 14 min. The activated gel was rapidly washed with 300 ml of cold water and 200 ml of 0.1 M sodium borate buffer, pH 9.5. The activated polymers (appeoximately 1 ml) were resuspended in 2 ml of 50 mM borate buffer, pH 9.5. Two mg of purified catalase were added directly to the gel suspension. The fixation was carried out at 4°C in a closed test tube with gentle stirring for 21 hours. The agarose derivative fixed human erythrocytes catalase were packed into a small column (0.6 cm×3.3 cm) and washed as follows : 0.1 M sodium borate buffer containing 1 M sodium chloride, pH 8.0 (8 ml); 0.1 M sodium acetate buffer containing 1 M sodium chloride, pH 4.0 (8 ml); the mentioned borate solution (10 ml); the mentioned acetate solution (10 ml); the mentioned borate buffer (8 ml); above acetate

buffer (8 ml); 0.1 M Tris-Cl buffer, pH 8.0 (102 ml) to break the activated sites by cyanogen bromide, and finally PBS (10 ml). The amount of human erythrocyte catalase fixed to agarose derivative was 1.76 mg (about 88% yield).

Purification of anti-human erythrocytes catalase antibody

Purification procedure was performed by an improved method of Karcher et al. (1972). The obtained antisera in rabbits were dialyzed against PBS. Solid ammonium sulphate was added to the dialyzed solution until the ammonium sulphate concentration reached 40% of saturation. The mixture was stirred at 4°C for 3 hours and then centrifuged at 8800×g for 30 min. The precipitate containing nearly all of the IgG was resuspended in 10 mM phosphate buffer, pH 6.8, and dialyzed with the same buffer.

A column (1.2 cm×12 vm) containing DE-52 was thoroughly equilibrated with the above mentioned dialysis buffer. The solution containing IgG was pumped through the column. IgG fraction containing anti-catalase antibodies did not bind to DE-52 at the experimental conditions. The pass-through fractions were gathered and dialyzed against PBS. The yield of the protein was about 75%.

Antigen affinity column

The dialyzed solution containing anti-catalase antibodies was applied to a human erythrocytes catalase binding CNBr-Sepharose CL-4B column. The column was washed with 30 ml of PBS. Anti-catalase antibodies were eluted from the column using 3 M Potasium thiocyanate. Fractions were collected and dialyzed against PBS. After antigen affinity column chromatography, the observed activity of the anti-catalase antibody was about 10 fold that of DE-52 fraction.

Preparation of anti-catalase antibody-alkaline phosphatase conjugate

The procedure was as described by the method of Van Weemen and Schuurs (1974) with some modifications. The mixture of nine hundred μg of the purified anticatalase-antibody in PBS and 1500 units of alkaline phosphatase in 3 M ammonium sulphate were dialyzed against PBS to remove ammonium sulphate. Five μl of 25% glutaraldehyde were added to 0.45 ml of the dialyzed solution and the mixture was incubated for one hour at 25°C and for 15 hours at 4°C. Unreacted glutaraldehyde was removed by dialysis with 0.1 M Tris-Cl buffer, pH 7.5. The conjugate was diluted with ELISA buffer (PBS containing 0.5% BSA, 0.05% Tween 20 and 0.02% sodium azide) of 5.4 ml, glycerol of 6 ml and magnesium chloride of 2 mg, and stored at −20°C until examination.

Assay of catalase content

The assay method was carried out by sandwich method (Belanger et al. 1973). The purified anti-catalase antibody of 1-0.05 μg in 100 μl of 0.1 M phosphate buffer containing 0.5% BSA, pH 8.0, was coated in each well of Nunc authorized immunoplate I. The plate was incubated at 4°C for 16 hours to 2 weeks or at −80°C for 6 weeks. Each well was washed three times with PBS containing 0.05% Tween 20. Appropriate dilutions of the purified catalase and erythrocyte

lysate with ELISA buffer were put in each well and incubated at 37°C for 1-4 hours. Each well was washed three times with PBS. One hundred μl of dilution conjugates (10-1000 fold) with ELISA buffer was added to each well and incubated at 37°C for 1-4 hours. These wells were washed with PBS four times. Two hundred μl of 2.5 mM p-nitrophenyl phosphate in 0.1 M carbonate buffer, pH 9.8, was added to each well and incubated at 37°C for 15-30 min. The reaction mixtures were diluted with the same buffer and their absorbances were measured at 405 nm with a Hitachi Model 200-20 Spectrophotometer.

Assay of catalase activity

Assay of catalase activity was performed by the method of Sinha (1972). The assay mixture consisted of 500 μl of 10 mM phosphate buffer pH 7.0, 100 μl of enzyme fraction and 400 μl of 0.2 M H_2O_2. The enzyme reaction was run at 25°C. Two ml of dichromate/acetic acid reagents was rapidly added to the reaction mixture and heated at 100°C for 10 min. After cooling, absorbance was measured at 570 nm with the spectrophotometer.

Calculation of Catalase activity

The velocity constant, K, was calculated from the first order reaction;

$$K = 1/t \log So/S$$

where So is the initial amount of H_2O_2 and S is the amount of H_2O_2 at t min. The value of $K(0)$ was calculated according to Ishikawa (1964) as follows:
Kat. $f = K(0)$/mg protein

Protein assay

Protein concentrations were determined by the method of Lowry et al. (1951).

Analytical polyacrylamide gel electrophoresis

Samples were electrophoresed on polyacrylamide disc gels at a constant current of 2 mA/gel in a pH 8.1 Tris/HCl buffer system. Protein was stained with Coomassie brilliant blue G-250 in 10% methanol by the method of Blackesly and Boezi (1977).

Iron determination

Iron concentration was determined with a Hitachi Atomic Absorption Spectrophotometer, Model 207.

Results and Discussions

Purification of catalase from human erythrocytes

Table 1 summarizes the purification of catalase from 500 ml of packed erythrocytes. The enzyme was purified approximately 1200-fold. It appeared to be homogeneous by judging from yielding a single band on polyacrylamide disc gel electrophoresis at pH 8.1. A ratio of absorbance at 280 nm per 405 nm was 0.805. It is consistent with the reports of Saha et al. (1964), and Stansell and Deutsch

Table 1. Purification of catalase from human erythrocyte. The results in the table represent typical value for purification of the enzyme from 500 ml of human blood.

step	volume (ml)	total protein (mg)	specific activity (K(O)/mg of protein)	fold	yield (%)
hemolysis	1,000	158,000	21	—	100
DE-52 1st	1,300	3,900	850	40	99.9
DE-52 2nd	350	577.5	4,842	231	84.3
60% $(NH_4)_2SO_4$ppt	—	50	18,791	895	28.3
Sephadex-G 200	88	41	25,274	1,204	31.2

(1965). Concentration of iron in our purified catalase was 1 μg/mg of protein. A ratio of iron/catalase was about 1/1000. This iron content (0.1%) in this investigation was in agreement with that, 0.098%, determined by other workers (Stansell and Deutsch, 1965; Bonnichen, 1947). The hemin concentration calculated on the basis of above mentioned iron content was 1.13%. The value was the same as the content indicated by Stansell and Deutsch (1965). These results suggest that erythrocytes catalase is completely purified by our method.

Antisera

All rabbits immunized with the human erythrocyte catalase produced antisera against catalase. One serum with the highest titer was chosen for purification of anticatalase-antibodies, and used for ELISA of catalase. The purified antibodies had high sensitivity and specificity compared with the antisera, since the antibodies were passed through an antigen (catalase) affinity column.

Determination of assay conditions

The dependency of the quantity of the purified anti-catalase antibody pre-coated in each well of an immunoplate was indicated Figure 2. In our assay procedure, more than 500 ng of antibodies pre-coated in a well was enough to bind the antigen of samples. One μg of the antibody was used for our ELISA. The pre-coated immunoplates were incubated at 37°C for 1-2 hours, at 4°C for 16 hours-2 weeks and at −80°C for 6 weeks (Figure 3). It was observed that the obtained standard curves were independent of the incubation time at this pre-coated step. The coated antibodies were stable at −80°C for 1.5 months and 4°C for 0.5 month. Large numbers of pre-coated plates were able to prepared and stored until examinations to simplify the procedure. The optimum incubation time for reactions of antibodies with antigen samples were 2 hours (Figure 4 a and b), the desired dilution of conjugates with ELISA buffer was 100-fold (data not shown). The assay conditions for alkaline phosphatase employed 2.5 mM p-nitrophenyl phosphate in 0.1 M carbonate buffer, pH 9.8 and 37°C at an incubation time of 30 min (data not shown).

Standard curves

A typical standard curve of the ELISA was shown in Figure 5. The minimum

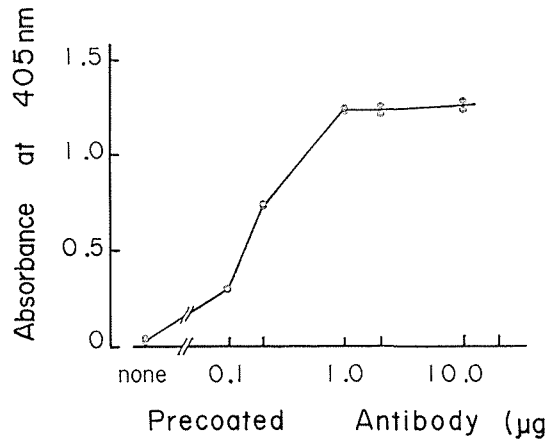

Figure 2. Optimization of purified antibody coating and tracer titration. Each amount of anti-catalase antibody was incubated at 4°C overnight in immunoplate wells. Each tracer dilution, containing 1 μg of purified catalase, was allowed to react with the absorbed antibodies. After washing, the 100 fold dilution of conjugates bound to antigen samples in wells at 37°C for 2 hours were determined by the adding 2.5 mM p-nitrophenyl phosphate substrate and incubating for 30 min at 37°C.

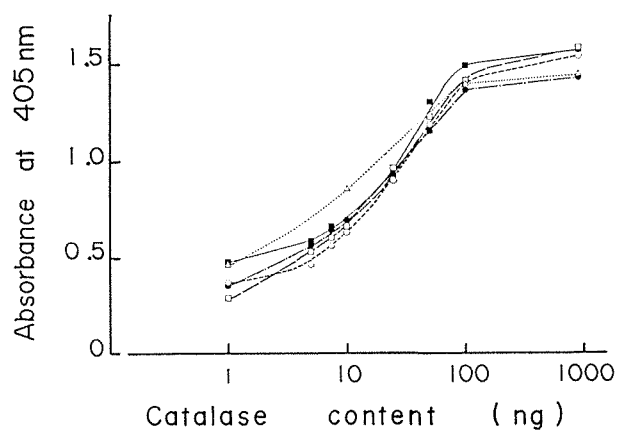

Figure 3. Comparison of dose response curves obtained by several incubation conditions of pre-coating antibodies. Each samples, containing various amount of purified catalase was react the absorbed one μg antibody at 37°C for 2 hours. The immunoassay was performed as described under "Methods" and "Figure 2". △·····△; at 37°C for one hour, ●-·-●; at 37°C for 2 hours, ○ - - ○; at 4°C overnight, ■——■; at 4°C for 2 weeks, and □ - - □; at −80°C for 6 weeks.

detectable content of catalase was 1 to 5 ng/well (4-20 fmol.). The standard curve was found to be linear in the range of 5-100 ng of catalase. It was confirmed that the curve was reproducible.

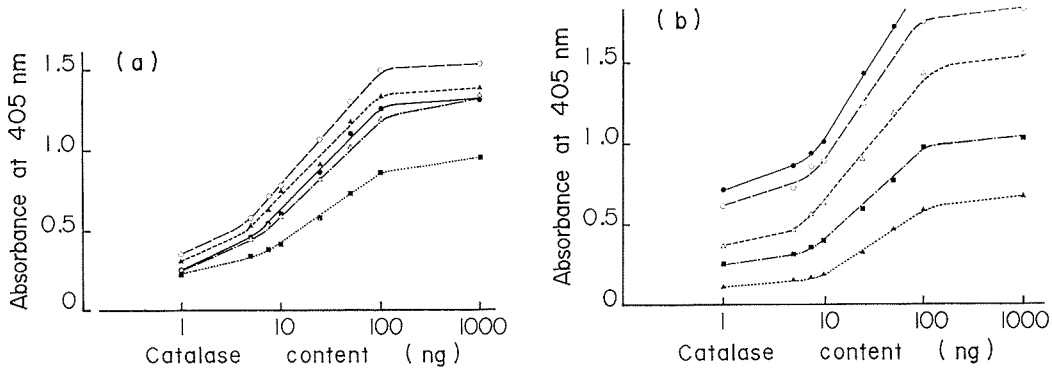

Figure 4. Comparison of dose response curves obtained by several incubation conditions of purified catalase as antigen and absorbed antibodies (a) and antigens and conjugates (b). in (a): ■·····■; at 37°C for 30 min, △-·-△; at 37°C for one hour, ○ - - ○; at 37°C for 2 hours, ▲ - - ▲; at 4°C overnight, ●——●; at 4°C for 24 hours, and in (b): ▲·····▲; at 37°C for 30 min, ■-·-■; at 37°C for one hour, △ - - △; at 37°C for 2 hours, ○ - - ○; at 37°C for 24 hours, ●——●; at 4°C for 24 hours.

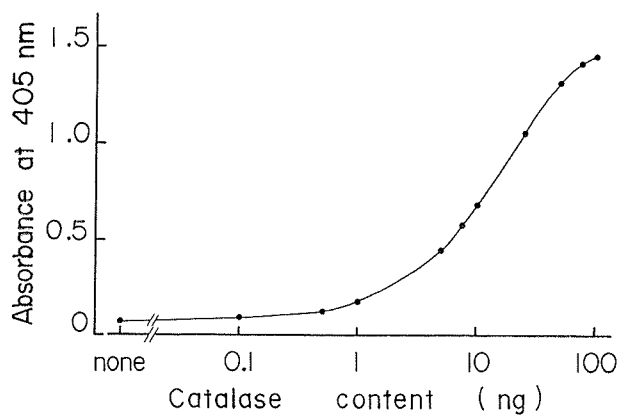

Figure 5. Standard curves for ELISA of catalase. The immunoassay was performed as described under "Figure 3".

Application of the ELISA to human erythrocytes

It is well known that sodium azide, aminotriazole and heat treatment inhibit catalase activity (Bhuyan and Bhuyan, 1977; Palcic and Dunford, 1981). In Figure 6-8, the effect of these inhibitors on the assay of catalase contents and activities was shown. Only after the heat treatment for samples containing the enzyme, catalase contents were not detected and its activities were abolished (Figure 6). These results seem to be caused by the denature of the enzyme. The other inhibitors had no effect on its contents although they inhibited enzyme as expected. Sodium azide, which was used in our ELISA assay system for preservation of the buffer containing BSA against decay, was observed to have no effect on this assay

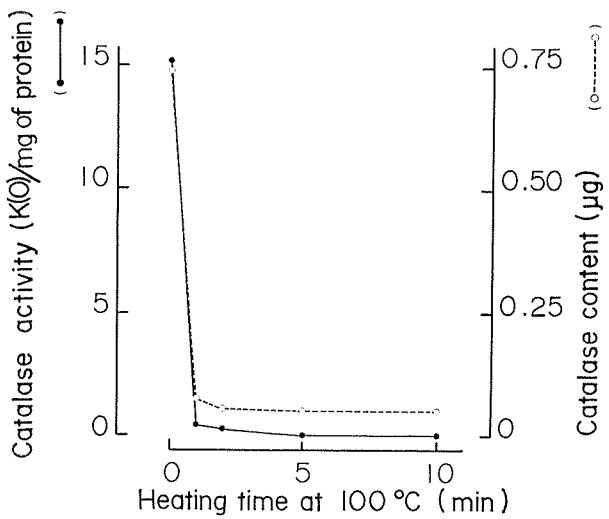

Figure 6. Inhibitory effects of a heat treatment on catalase activities and content. Activities and contents are expressed as (●—●): catalase activity, (○···○): catalase content. The heat treatment was added to samples and then, they were assayed in two methods were described in the text.

Table 2. The content of catalase in human eryhtrocytes of normal adults

	NaN_3 treatment	mean content (μg/mg of Hb)	range
Normal adults (n=5)	−	2.37	1.77—2.54
Normal adults (n=5)	+	2.32	1.99—2.57

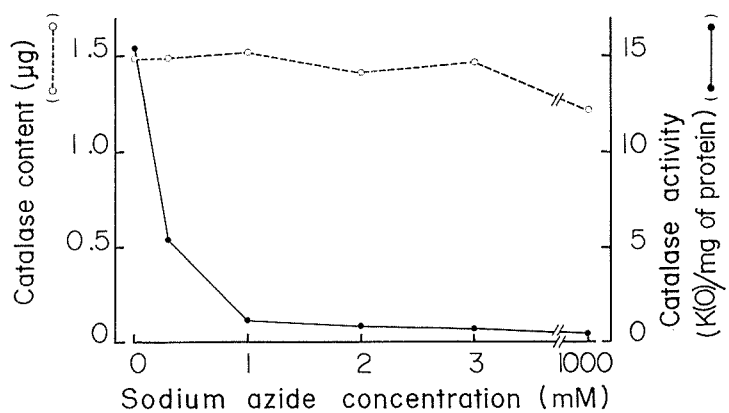

Figure 7. Inhibitory effects of sodium azide on catalase activity and content. Activity (●—●) and content (○···○) are expressed. Sodium azide was added to samples and they were preincubated for 30 min at 25°C, then assayed by the two methods were described in the text.

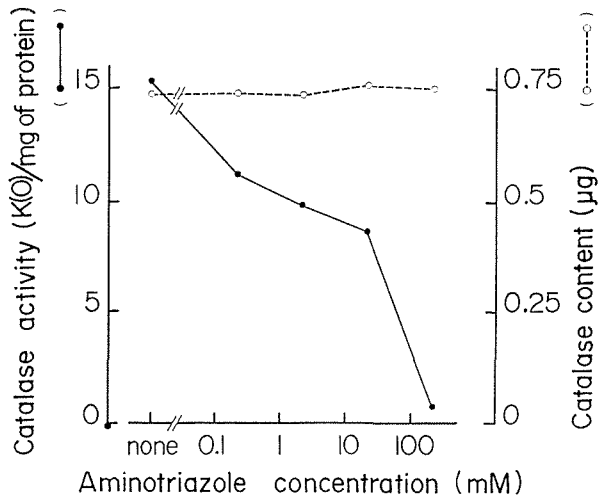

Figure 8. Inhibitory effects of aminotriazole on catalase activity and content. Activity (●—●) and content (○···○) are expressed. Aminotriazole was added to samples and they were preincubated for 30 min at 25°C, then assayed by the twe methods were described in the text.

(Figure 7, Table 2). These results suggest that inactive forms of the enzyme may be detected, and that the levels remain constant independent of enzymatic inactivation. It has been reported that in various diseases, such as acatalasemia and leukemia, erythrocytes catalase presents partial activity (Saito et al., 1984; Ogata et al., 1975). It is determined that deficiency of catalase is caused by whether its low contents or inactive form by the ELISA. Thus the described ELISA for catalase determination is applicable to studies of enzyme concentration in clinical situations.

Direct measurements of catalase contents by the ELISA were carried out on erythrocyte fractions in human blood. Human erythrocytes were separated from whole blood by centrifugation and washing. These samples were diluted 2500 to 5000-fold with the ELISA buffer. The results were shown in Table 2. The mean value was 2.37 μg/mg of Hb. This value was higher than that (1.95 μg per mg of haemoglobin) determined by Ben-Yoseph and Shapira (1966), or that (0.78 μg per mg of erythrocytes) reported by Hartz et al. (1973), or that (1.37 μg per mg of haemoglobin) measured by Stansell and Deutsch (1966). In this paper, the erythrocyte catalase level which might be due to high sensitivity of the ELISA was similar to, or slightly higher than, the reported levels. One main advantage of this ELISA for catalase developed for the first time in our laboratory is the simplicity of its performance. A comparatively large number of erythrocytes samples can be rapidly examined by this simple technique, and the content of catalase in various physiological and pathological conditions are revealed.

References

Axen, R. and Ernback, S. (1971): Chemical fixation of enzymes to cyanogen halide activated polysaccharide carriers. Eur. J. Biochem. 18, 351–360.

Belanger, L., Sylvestre, C. and Dufour, D. (1973): Enzyme-linked immunoassay for alpha-fetoprotein by competitive and sandwich procedures. Clin. Chim. Acta **48**, 15–18.

Ben-Yoseph, Y. and Shapira, E. (1973): Specific immunoassay for quantative determination of human erythrocyte catalase. J. Lab. Clin. Med. **81**, 133–139.

Blakesly, R. A. and Boezi, J. A. (1977): A new staining technique for protein in polyacrylamide gel using coomassie brilliant blue G 250. Anal. Biochem. **82**, 580–582.

Bhuyan, K. C. and Bhuyan, D. K. (1977): Regulation of hydrogen of peroxide in eye humors. Biochem. Biophys. Acta **497**, 641–651.

Bonnichen, R. 1947, in Methods in enzymology (Colowick, S. P. and Kaplan, N. O., eds) Vol. II, p. 816.

Cuatrecasas, P. (1970): Protein purification by affinity chromatography. J. Biol. Chem. **245**, 3059–3065.

Geerts, A. and Roels, F. (1982): In vivo cooperation between hepatic catalase and superoxide dismutase demonstrated by diethyl-dithiocarbamate FEBS Lett. **140**, 245–247.

Hartz, J. W., Funakosi, S. and Deutsch, H. F. (1973): The levels of superoxide dismutase and catalase in human tissues as determined immunochemically. Clin. Chim. Acta **46**, 125–132.

Ishikawa, Y., Tamada, S. and Ariga, F. (1964): Clinical significance of successive estimation of the blood catalase activity in the cancer patient during surgical and anticancer drug therapy. Tohoku J. exp. Med. **34**, 81–91.

Kaji, H., Kurasaki, M., Ito, K., Saito, T., Saito, K., Niioka, T., Kojima, Y., Ohsaki, Y., Ide, H., Tsuji, M., Kondo, T. and Kawakami, Y. (1985): Increased lipoperoxide value and glutathione peroxidase activity in blood plasma of type 2 (non-insulin-dependent) diabetic woman. Klin. Wochenschr. **63**, 765–768.

Karcher, D., Matthyssens, G. and Lowenehal, A. (1972): Isolation and characterization of IgG globulins in subacute sclerosing panencephalitis. Immunology **23**, 93–99.

Kurasaki, M., Ito, K., Saito, T. and Saito, K. (1982): Human erythrocyte catalase activity in Japanese adults. Jpn. J. Hyg. **37**, 707–713.

Kurasaki, M., Saito, T., Kaji, H., Kojima, Y., and Saito, K. (1986): Increased erythrocytes catalase activity in patients with hyperthyroidism. Horm. metab. Res. **18**, 56–59.

Lal, A. J., Ansari, N. H. and Awasthi, Y. C. (1980): Defense of mouse red blood cells against oxidative damage by phenylhydrazine. J. Lab. Clin. Med. **95**, 536–552.

Lowly, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Mancini, G., Carbonera, A. O. and Heremans, J. F. (1965): Immunochemical quantitation of antigens by single radial immunodiffision. Immunochemistry **2**, 235–254.

Ogata, M. Mizugaki, J. (1979): Properties of residual catalase in the erythrocytes of Japanese acatalasemia. Hum. Genet. **48**, 329–338.

Palcic, M. M. and Dunford, H. B. (1981): The kinetics of cyanide binding by human erythrocyte catalase. Arch. Biochem. Biophys. **211**, 245–252.

Saha, A., Campbell, D. H. and Schroeder, W. A. (1964): Immunochemical studies on liver and erythrocytes catalase from cattle, horse, rabbit and human. Biochim. Biophys. Acta **85**, 38–49.

Saito, T., Ito, K., Kurasaki, M., Fujimoto, S., Kaji, H. and Saito, K. (1982): Determination of true specific activity of superoxide dismutase in human erythrocytes. Clin. Sci. **63**, 251–255.

Saito, T., Kurasaki, M., Kaji, H. and Saito, K. (1984): Deficiency of erythrocyte superoxide dismutase and catalase activities in patients with malignant lymphoma and acute myeloid leukemia. Cancer Lett. **24**, 141–146.

Sinha, A. K. (1972): Colormetric assay of catalase. Anal. Biochem. **47**, 389–394.

Stansell, M. J. and Deutsch, H. F. (1966): The levels of catalase and of erythrocupprein in human erythrocytes. Clin. Chim. Acta **14**, 598-607.

Stansell, M. J. and Deutsch, H. F. (1965): Preparation of crystalline erythrocuppreine and catalase from human erythrocytes. J. Biol. Chem. **240**, 4299-4305.

Van Weemen, B. K. and Schuurs, A. H. W. M. (1974): Immunoassay using antibody-enzyme conjugates. FEBS Lett. **43**, 215-218.

(Received 30, August 1986)