Vol. 19, No. 5, November 1989 BIOCHEMISTRY INTERNATIONAL
Pages 1117-1124

SUPEROXIDE - DRIVEN OXIDATION OF QUERCETIN AND A SIMPLE SENSITIVE ASSAY FOR DETERMINATION OF SUPEROXIDE DISMUTASE

Vladimir A. Kostyuk and Alla I. Potapovich

Laboratory of Bioenergetics, Department of Biology, V.I.Lenin Byelorussion State University, Minsk, BSSR, USSR

Received May 6, 1989

Summary. Oxidation of quercetin at pH 10 was shown to be a free radical chain reaction involving superoxide and hence inhibitable by superoxide dismutase (SOD) (EC 1.15.1.1). The degree of inhibition of quercetin oxidation was a function of SOD concentration, and fifty percent inhibition was produced by approximately 1.5 ng/ml of pure enzyme. This reaction proved to be a very useful tool for a rapid and highly sensitive measurement of SOD in crude tissue extracts and other biological samples.

Introduction

Many investigators in the field of biochemistry, plant physiology, and clinical chemistry are faced with the need to assay superoxide dismutase (SOD) (EC 1.15.1.1). The direct measurement of SOD activity is possible (1), but the procedure requires special apparatus usually lacking in the typical biochemical laboratory. Therefore a variety of methods based on the inhibition of superoxide - driven reactions by SOD has been developed. Numerous such reactions and enzymic or non - enzymic sources of superoxide are known at present (2, 3). Substances which can act both as the source of superoxide and as the indicating scavenger for this radical have been found(4, 5). Nevertheless these methods have certain limitations and the need still exists to increase the accuracy, specificity, and simplicity of the assay.

A new simple assay for superoxide dismutase was developed by adopting a purely chemical reaction of quercetin oxidation at pH 10 in the presence of N, N, N', N'- tetramethylethylenediamine (TMEDA). This procedure, involving rather stable and commonly available reagents, allows a rapid and highly sensitive measurement of SOD in crude tissue extracts and other biological materials.

0158-5231/89/111117-08$01.00/0

Materials and Methods

Chemicals. Quercetin and sodium azide were purchased from Sigma Chemical Company; superoxide dismutase was from Toyobo Co., Ltd., Osaka; catalase was from Serva, Heidelberg; TMEDA and nitro blue tetrazolium (NBT) were from Reanal, Budapest.

Stock solutions were prepared with distilled water: (1) 0.02 M phosphate buffer (pH 7.8); (2) 5 mM TMEDA, 0.5 mM EDTA solution.

Autoxidation. Quercetin was autoxidized at room temperature under normal fluorescent laboratory lighting. The standard incubation mixture contained 0.016 M phosphate buffer, 0.8 mM TMEDA, and 0.08 mM EDTA in total volume 3.0 ml. The final pH of the reaction medium was 10. The reaction was initiated by the addition of 0.1 ml of quercetin stock solution (1.5 mg in 10 ml of N, N - dimethylformamide) and followed by measuring absorbance at 406 nm.

Equipment. Changes in absorbance were recorded with Specord M-40 UV-VIS spectrophotometer.

Results and Discussion

Autoxidation of quercetin. A vigorous quercetin oxidation was found at pH 10 in the presence of 0.8 mM TMEDA and 0.08 mM EDTA. The reaction was accompanied by a loss of the 406-nm absorption band of quercetin and an increase in absorbance at 320 nm with an isosbestic point at 380 nm (Fig.1). The rate of quercetin oxidation sharply decreased with decreasing pH over the range of 10 to 8.0 and also fell sharply with decreasing concentration of TMEDA (data not presented). The change in absorbance at 406 nm was linear for a period of 20 min (Fig.2). Therefore in all following experiments the length of incubation for quercetin oxidation was 20 min.

To determine activated oxygen species involved in the reaction of quercetin oxidation, we used SOD, catalase, and sodium azide and found that neither catalase (0.2-20 mg/l) nor sodium azide (6-60 mM) inhibited quercetin oxidation. These findings therefore demonstrate that singlet oxygen, H_2O_2, and consequently $\cdot OH$ are neither initiators nor critical intermediates in this reaction. In contrast, the addition of SOD to the incubation mixture caused a proportionate inhibition of the rate of quercetin oxidation (Fig.3), confirming the involvement of superoxide in this process.

Also, the appearance of formazan was found during a joint in-

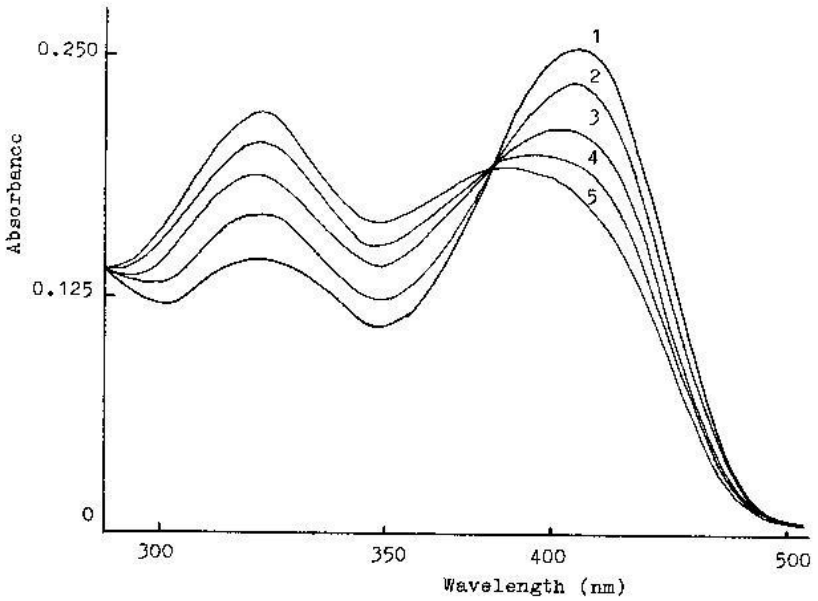

Fig. 1. Oxidation of quercetin (0.014 mM) at pH 10 in the presence of 0.8 mM TMEDA and 0.08 mM EDTA. 1-5, respective absorption spectra 30, 300, 600, 900, and 1200 s after mixing the reactants.

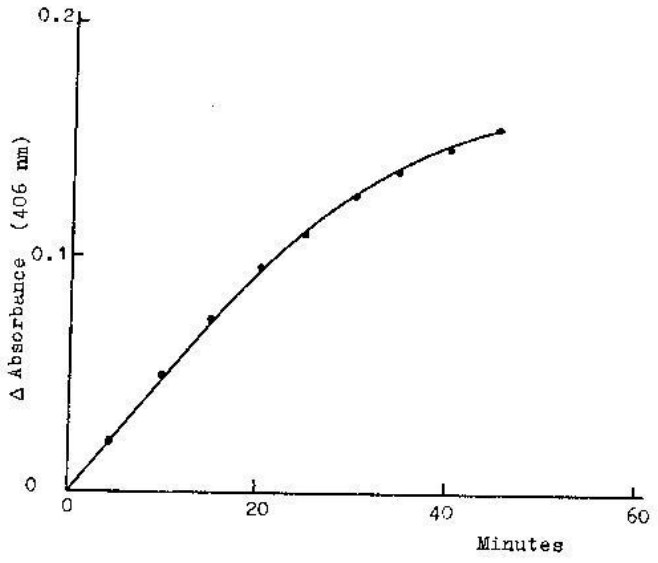

Fig. 2. Time - course of the reaction of quercetin (0.014 mM) oxidation at pH 10 in the presence of 0.8 mM TMEDA and 0.08 mM EDTA.

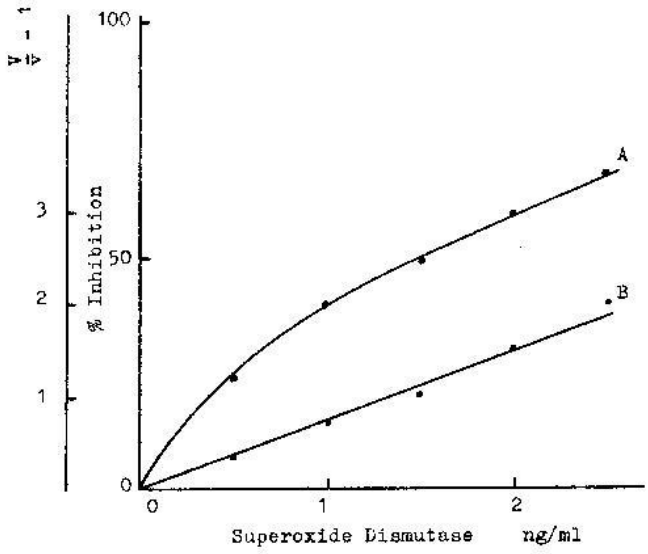

Fig. 3. Cu, Zn SOD - induced inhibition of the oxidation of quercetin. Experimental procedures were described in Materials and Methods. (A) dose - response curve for the effect of SOD; (B) linearization of the curve. V and v are, respectively, the changes in absorbance (406 nm) in the absence and in the presence of SOD. Values represent the average of 3 separate determinations.

cubation of NBT, an indicator scavenger for superoxide, with quercetin (Table I). Quercetin does not reduce NBT directly since this reaction occurred more slowly under partialy anaerobic conditions (5-min bubbling with nitrogen) and was almost completely inhibited by SOD (Table I). The more higher concentration of SOD in this case is apparently due to competition of the enzyme with NBT for superoxide. These results provide further support for the involvement of superoxide in quercetin oxidation.

Recently a mutagenic action of quercetin was found (6, 7). It seems likely that this phenomenon may be related to the ability of quercetin to generate superoxide. Anyway, rutin, of which quercetin is the aglycone, has no mutagenic effect and can not generate superoxide during autoxidation (data not shown).

Determination of SOD in biological materials. Any reaction inhibitable by SOD could be potentially used as the basis for an assay of this dismutase, but only those reactions which are the

Table 1. Appearance of formazan (ΔA_{515}) during a joint incubation of NBT (0.5 mM) with quercetin (0.014 mM)

Additions	ΔA_{515}/10 min
NBT alone	0.009
quercetin alone	0.000
NBT + quercetin	0.090
NBT + quercetin + SOD (150 ng/ml)	0.017
NBT + quercetin (partialy anaerobic)	0.054

Reaction mixture contained 0.016 M phosphate buffer, 0.8 mM TMEDA, 0.08 mM EDTA. The total volume was 3.0 ml.

most sensitive to the SOD action would permit reliable determination of the enzymatic activity in tissue extracts, especially in extracts with low SOD activity. The sensitivity of an indirect assay can be defined in terms of the concentration of SOD that inhibits a superoxide - driven reaction by 50% (I_{50}). Using the dose-response curve for the effect of SOD on quercetin oxidation (Fig.3), we found that fifty percent inhibition, i.e., I_{50}, is produced by 1.5 ng/ml of pure enzyme; while the values of I_{50} for epinephrine (4) and NADH (5) oxidation are 50 and 15 ng, respectively.

The reaction of quercetin oxidation was used to determine SOD levels in tissues and body fluids (Table 2). To perform the assay, we added sequentially the following (see Materials and Methods) to a tube: phosphate buffer, 2.5 ml; TMEDA/EDTA solution, 0.5 ml; sample (or water), 0.1 ml. After thorough mixing, 0.1 ml of quercetin stock solution was added and the decrease in absorbance at 406 nm was followed for 20 min.

The dose - response curve of SOD inhibition of quercetin oxidation or its linearization by the method of Asada et al. (8) can be used as the calibration curves up to 65-70% of the inhibition (Fig.3).

Due to the high sensitivity of the method, samples of tissue extracts and blood can be diluted with water by such a large fac-

Table 2. SOD levels in body fluids and tissues

Fluid or tissue tested	Preliminary dilution	Levels of SOD μg/ml of blood (or μg/g of tissues)
Human blood (Adult) *		
Male	1000	31.0 ± 2.8
Female	1000	21.0 ± 1.9
Human blood plasma	10	0.23± 0.06
Human synovial fluid (Rheumatoid patients)	10	0.27± 0.07
Blood of female white rats *	1000	34.5 ± 2.45
Brain of female white rats **	1000	52.5 ± 3.8
Liver of female white rats **	2000	197.0 ±10.1

* - whole blood diluted with water;
** - 10% (w/v) tissue homogenate in 0.9% NaCl diluted with water

tor (Table 2) that any nonenzyme interfering activity is negligible. The dose - response curve for the effect of crude enzyme preparations was essentially identical to the calibration curve, and boiled or protein free samples (filtration with membrane filter CF25) failed to cause any inhibition of quercetin oxidation (Fig.4).

On the contrary, synovial fluid and blood plasma are usually diluted only 10-fold, and boiled samples have a poor activity. This activity can be related to low molecular weight scavengers of free radicals, for example, ascorbic acid. Indeed, we have found that the presence of 10 and 1 μM ascorbic acid inhibits quercetin oxidation by 80 and 45%, respectively. To avoid all interference by compounds mentioned above, samples can be dialyzed against suitable media or desalted using a small Sephadex G - 25 column.

Besides low molecular weight scavengers and SOD, crude samples from body fluids and tissues may include proteins that are capable of removing superoxide, for example, metal transport proteins. Their influence on SOD determination, however, seems to be negligible because blood plasma, containing considerable amount

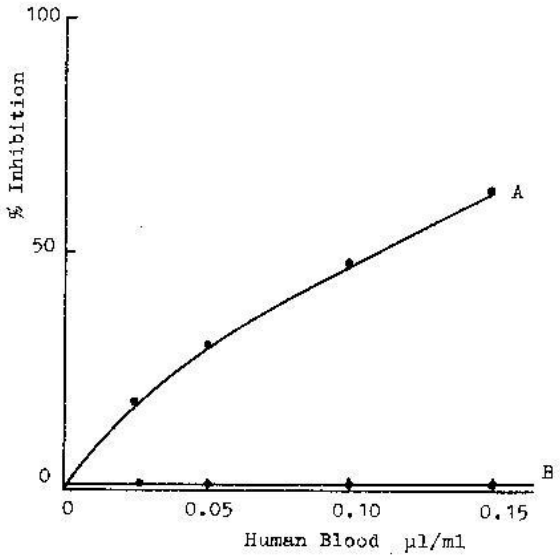

Fig. 4. Inhibition of the oxidation of quercetin by SOD in human blood. Experimental procedures were described in Materials and Methods. (A) native samples; (B) boiled (15 min) or filtered (membrane filter CF25) samples. Values represent the average of 3 separate determinations.

such proteins, has very poor ability for inhibiting quercetin oxidation (Table 2). Hemoglobin at the concentration corresponding to its amount in diluted blood samples also does not inhibit quercetin oxidation.

In conclusion, the reaction of quercetin oxidation was shown to be the basis for a sensitive and reliable assay for SOD involving stable and commonly available reagents and consisting of a single spectrophotometric step.

References

1. Marklund, S.L., Bjelle, A., and Elmqvist, L.G. (1986) Ann. Rheum.Dis. 45, 847-851.
2. Beyer, W.F., Jr. and I. Fridovich (1987) Anal.Biochem. 161, 559-566.
3. Flohe, L., and Otting, F. (1984) in Methods in Enzymology (Colo-wick, S.P., and Kaplan, N.O., eds.) Vol. 105, pp. 93-104, Academic Press, New York.

4. Misra, H.P., and Fridovich, I. (1972) J. Biol.Chem. 247, 3170-3175.
5. Paoletti, F., Aldinucci, D., Mocali, A., and Caparrini, A. (1986) Anal.Biochem. 154, 536-541.
6. Jose, R., Laires, A., Borba, H., Chaveca, T., and Gomes, M.I. (1986) Mutagenesis 1, 179-184.
7. Rastogi, P.B., and Levin, R.E. (1987) Environmental Mutagenesis 9, 79-87.
8. Asada, K., Takahashi, M., and Nagate, M. (1974) Agric.Biol. Chem. 38, 471-473.