IgG antibodies with peroxidase-like activity from the sera of healthy Wistar rats

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Abstract Various catalytic antibodies or abzymes (Abzs) have been detected recently in the sera of patients and animals with many autoimmune diseases, where their presence is most probably associated with autoimmunization. Normal humans or animals usually do not contain Abzs. In contrast, polyclonal Abzs from healthy humans and animals have an intrinsic superoxide dismutase activity and catalyze formation of H_2O_2 (Wentworth et al., 2000, *Proc. Natl. Acad. Sci. USA*; 2001, *Science*). Here, we present the first evidence showing that highly purified native IgGs from the sera of healthy Wistar rats interact with H_2O_2 and possess peroxidase-like activity. Specific peroxidase activity of IgG preparations from the sera of 10 rats varied in the range 1.6–27% as compared with that for horseradish peroxidase (100%). Antioxidant enzymes such as superoxide dismutases, catalases, and glutathione peroxidases are known to represent critical defence mechanisms for preventing oxidative modifications of DNA, proteins, and lipids. Antioxidant peroxidase activity of Abzs can also play an important role in the protection of organisms from oxidative stress as well as in oxidation of toxic compounds.
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1. Introduction

At present, artificial and natural abzymes (Abzs) catalyzing more than 100 distinct chemical reactions have been described (reviewed in [1–7] and references therein). The first example of a natural Abz hydrolyzing vasoactive intestinal peptide was an IgG found in bronchial asthma patients [8]. This was followed by a discovery of IgG with DNase [9] and RNase activities in systemic lupus erythematosus [10]. Later, IgGs and/or IgMs

hydrolyzing RNA, DNA, peptides, proteins, or polysaccharides have been described in the sera of patients with several autoimmune (AI) pathologies (for a review, see [4–7]). It is shown that catalytic Abzs in principle can be produced not only in the organisms of AI patients, but also in the healthy humans. However, with some exceptions of Abzs hydrolyzing peptides [11,12], catalytic activities of Abs of healthy volunteers are usually very low. As an example, we did not detect DNase and RNase Abzs in the serum samples from 50 healthy volunteers or from patients with many different diseases proceeding without significant AI reactions [4–7]. Polysaccharide-hydrolyzing activity in healthy donors was ~40–100 times lower than that in AI patients [13,14].

All higher organisms generate energy by aerobic respiration, a process that involves a stepwise four-electron reduction of molecular oxygen to water [15–17]. The partially reduced species that are produced as intermediates and by-products of aerobic respiration, including $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$, are potent oxidants attacking different cellular DNA, proteins and lipids [15]. These oxidants also appear in cells through exposure to ionizing radiation and agents that generate free radicals. Oxidative damage to the cell is ongoing and has been regarded as a significant factor in carcinogenesis and aging [16,17]. Therefore, special attention had been focused on understanding the mechanism of oxidative stress and oxidative damage to DNA, lipids, and proteins.

Antioxidant enzymes such as superoxide dismutases, catalases, and glutathione peroxidases represent critical defence mechanisms for preventing oxidative modifications of DNA, proteins and lipids [18,19]. Therefore, it is very interesting whether only these enzymes possess antioxidant activity or Abs can also play an important role in the protection of organisms from oxidative stress.

As was shown recently, mono- and polyclonal Abs from different sources have an intrinsic ability to intercept singlet $^1O_2$ and efficiently reduce it to $O_2^{\bullet}$ [20,21]. These intact Abs were shown to catalyze formation of hydrogen peroxide in a catalytic process, and the electron source for a quasi-unlimited generation of H_2O_2 was identified [21]. From isotope incorporation experiments and kinetic data, Abs were shown to use H_2O as an electron source, facilitating its addition to $^1O_2^{\bullet}$ to form H_2O_3 as the first intermediate in a reaction cascade that eventually leads to H_2O_2. These findings suggest a protective function of Abs against $^1O_2^{\bullet}$ and raise the question of whether

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Abbreviations: Abs, antibodies; Abzs, abzymes, or catalytically active antibodies; AI, autoimmune; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; SDS–PAGE, SDS–polyacrylamide gel electrophoresis

the need to detoxify $^1O_2^*$ has played a decisive role in the evolution of the immunoglobulin fold [20,21]. Thus, Abzs offer a mechanism by which oxygen can be reduced and recycled during phagocyte action, thereby potentiating the microbial action of the immune system [21]. The question whether Abzs with catalase or peroxidase like activities converting H_2O_2 exist in mammalian sera, however, remains open.

As we have shown recently, Wistar rat strain is a good model for studying repair and antioxidant enzymes [22]. In the present study, we have used different approaches to provide a convincing evidence that peroxidase-like activity is intrinsic to rat IgGs.

2. Materials and methods

Reagents used in this work were obtained mainly from Merck and Sigma. The sera of 10 healthy Wistar rats (6–8 months old) were used to search for Abzs. Electrophoretically homogeneous IgGs were obtained by sequential affinity chromatography of rat serum proteins on protein A-Sepharose (Sigma) and by FPLC gel filtration on Superdex 200 HR 10/30 (Pharmacia) under "acid shock" conditions as described previously [23–26].

In order to analyze metal ions bound to Abs, IgGs purified on Protein A-Sepharose was reloaded on the Protein A-Sepharose (1 ml) equilibrated with 50 mM Tris–HCl (pH 7.5), the column was washed with the same buffer and metal ions were eluted with buffer 25 ml of 50 mM Tris–HCl (pH 7.5) containing 50 mM EDTA; the eluate was lyophilized. The same amount of control lyophilized powder was prepared from 25 ml of the buffer passed through the column free of Abs. All powders (5–7 mg per one analysis) were used for metal composition analysis by two-jet arc plasmatron atomic emission technique according to [27,28]. The mass percentage of metals (% of powder containing all dried components of eluate) was determined. Final value of mass percentage of each metal was estimated from a difference between the corresponding experimental and control powder samples.

Isolation of IgG fraction bound to metal ions was performed on a Chelex-100 column (7×20 mm) free of metal ions and equilibrated in H_2O. IgG (~3 mg) was loaded on the column, which then was stepwise washed with 3 ml of 10 mM Tris–HCl (pH 7.5), the same buffer containing 0.5 M NaCl, and 1.0 M NaCl. Optical density and catalytic activity were measured in all fractions.

Catalase and glutathione peroxidase activities were assayed as described in [29].

Measurements of the IgG peroxidase activity was carried out using the optimal conditions: 25 mM potassium phosphate (pH 6.8), 0.1 M KCl, 10 mM H_2O_2, 0.05–0.6 mg/ml 3,3′-diaminobenzidine, and 0.01–0.3 mg/ml IgGs. Horseradish peroxidase activity was measured using the same conditions and 1–3 μg/ml of HRP. The reactions (100 μl total volume) were performed in multiwell plates in the dark at 22 °C for 0.5–10 min. Optical density (A_{450}) of the solutions was determined using a Labsystems Uniskan II spectrophotometer. Reaction mixture containing no Abs or HRP was used as control. All measurements (initial rates) were taken within the linear regions of the time courses and Ab concentration curves.

SDS–PAGE analysis of IgG under reducing and non-reducing conditions was performed in 4–15% gradient gels (0.1% SDS) according to Laemmli with silver staining [23–26]. SDS–PAGE analysis of peroxidase-like activity of IgGs was performed similarly to [19–22]. To restore the enzymatic activity after SDS–PAGE, SDS was removed by incubating the gel for 1 h at 22 °C in potassium phosphate (pH 6.8) and washing the gel five times with the same buffer. To allow protein refolding and to assay for peroxidase activity, longitudinal slices of the gel were incubated in the standard reaction mixture containing 10 mM H_2O_2 and 0.2 mg/ml DAB. The parallel longitudinal slices were used for detecting the position of IgG on the gel by Comassie R250 staining.

The K_m and V_{max} values were calculated from the kinetic data by least-squares non-linear regression fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver–Burk plot [30]. Errors in the values were within 10–30%.

3. Results and discussion

To search for the Abzs in the rat sera, the IgG fraction was purified by chromatography on Protein A-Sepharose under special conditions to remove non-specifically bound proteins [23–26]. Strong non-covalent protein complexes usually dissociate under acidic conditions. The purified IgG was incubated at pH 2.6 to dissociate non-covalent interactions and ensure that other proteins were not tightly bound to it, resulting in three peaks corresponding to IgG, IgA, and IgM after FPLC gel filtration in the acidic buffer (pH 2.6). The homogeneity of typical 150 kDa IgG was confirmed by SDS–PAGE with silver staining, which showed a single band under non-reducing conditions and two bands corresponding to the H and L chains after the reduction (Fig. 1).

We have first analyzed catalase and glutathione peroxidase activities of purified IgGs, which are typical activities for human blood enzymes. However, both of these activities in the case of IgGs from ten different rats were very low, on borderline of the sensitivity of the methods used. Some plants are known to contain specific enzymes with peroxidase activity, such as horseradish peroxidase [31]. HRP forms a complex with H_2O_2 and then one-electron reduction of the second substrate takes place [31]. Peroxidases of this type can utilize different organic compounds as the second substrate, including 3,3′-diaminobenzidine (DAB), which is one of the best HRP substrates. All ten IgG preparations demonstrated high peroxidase activity (see below).

To prove that peroxidase activity of IgG is intrinsic property of the Abs and is not due to co-purifying enzymes, we have applied several strict criteria. They may be summarized as follows: (a) Abs were electrophoretically homogeneous (Fig. 1); (b) FPLC gel filtration of IgGs under conditions of dissociation of strong non-covalent complexes (3 M $MgCl_2$) or in the acidic buffer (pH 2.6, Fig. 2A) did not lead to disappearance of the activity, and the peak of activity tracked exactly with complete IgG form; (c) immobilized rabbit polyclonal IgGs against rat IgGs completely absorbed the peroxidase activity, which can be found then only in the peak of IgGs eluted by acidic buffer (Fig. 2B).
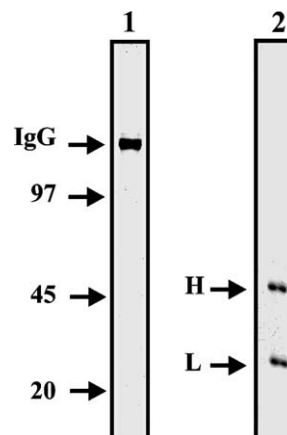

Fig. 1. SDS–PAGE in a non-reducing 4–15% gradient gel (lane 1) and in a reducing 12% gel (lane 2) followed by silver staining of IgG (5 μg after FPLC gel filtration). H, immunoglobulin heavy chain; L, immunoglobulin light chain. Arrows indicate the positions of molecular weight markers.
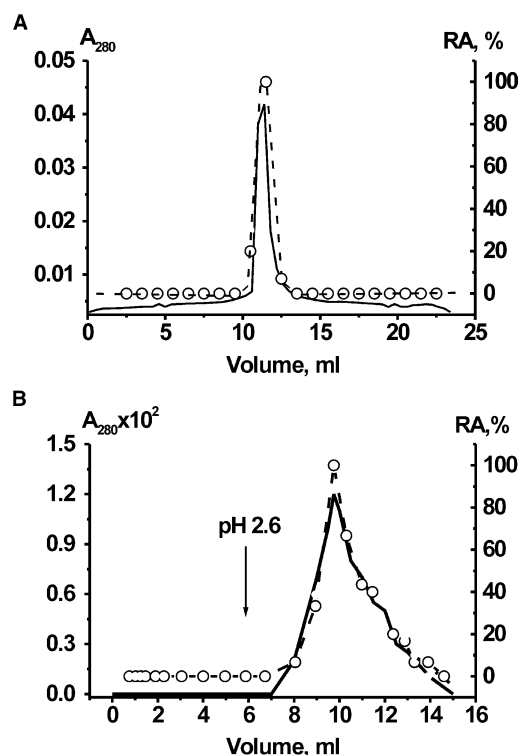
Fig. 2. FPLC gel filtration of IgG on a Superdex 200 column under acidic conditions (pH 2.6) after Abs incubation in the same buffer (A) and affinity chromatography of IgG on Sepharose bearing rabbit IgGs against rat IgGs (B): (–), absorption at 280 nm, (O), relative peroxidase activity of IgGs (RA). RA of the fractions with the highest activity was taken for 100%.

Fig. 3. In situ SDS–PAGE analysis of rat IgG peroxidase activity. After the electrophoresis, the gel was incubated under special conditions for protein refolding. Then the relative peroxidase activity was revealed by incubating longitudinal gel slices in the reaction mixture containing H_2O_2 and DAB (lane 1). An additional control longitudinal slice of the same gel was stained with Comassie R250 to reveal the positions of IgG (lane 2). Arrows indicate the positions of molecular weight markers.

To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the Abs were subjected to SDS–PAGE and their peroxidase activity was detected by incubation of the gel in the standard reaction mixture containing H_2O_2 and DAB. A yellow-brown band was revealed only in the position of IgG (Fig. 3). Since SDS dissociates all protein complexes, the detection of the activity in the gel region corresponding only to IgG, together with the absence of any other bands of the activity or protein (Fig. 3), provides a direct evidence that IgG possesses this activity.

The most known plant, bacterial and mammalian oxidases, peroxidases and dismutases converting active forms of oxygen are metal-dependent enzymes. For example, plant HRP-like peroxidases and mammalian catalases are Fe^{2+}-porphyrin dependent enzymes [31,32]. Two superoxide dismutases from mammals are Mn- and Cu,Zn dependent, while in some bacteria Ni- and Fe-dependent enzymes are found [32].

It was interesting whether rat peroxidase IgGs are also metal-dependent enzymes. In order to find out what metal ions are complexed with purified Abs, we have reapplied previously purified IgGs to protein A-Sepharose and then metal ions bound to IgGs adsorbed by this resin were eluted in 50 mM Tris–HCl (pH 7.5) containing 50 mM EDTA. The control eluate was obtained by passing the same buffer through protein A-Sepharose free of IgGs, and both eluates were lyophilised. The difference in the composition of metal ions was analyzed in the lyophilized experimental and control powders by two-jet arc plasmatron atomic emission as in [27,28]. This method a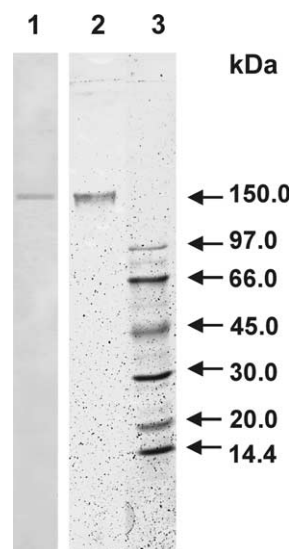llows determination of only the types and the relative amounts of different metals but provides no information on the charges of the ions analyzed. The following metals were revealed to be bound to IgGs from the sera of two rats: Cu > Fe > Ni $\geqslant$ Co $\geqslant$ Mn. Interestingly, the relative amount of different metal ions which can potentially participate in oxidation reactions bound to IgGs from the sera of two rats differed from ~3-fold for Mn up to ~8-fold for Fe. The relative amount of Cu was about 17–194-fold higher than that for Ni or Co.

Metal dependent enzymes usually have affinity to Me-ions chelating adsorbents. Therefore we have tried to separate IgGs bound to metal ions from Abs containing no ions on Chelex-100. In order to analyze an average situation we have loaded the Chelex column with a mixture of equal amounts of IgGs from the sera of four rats. The IgGs corresponding to the main first peak (except its tail, see Fig. 4) with no affinity for the chelating resin (86–87% of the total protein) did not possess detectable peroxidase activity. Approximately 13–14% of IgGs were adsorbed by the resin and eluted with 0.5–1.0 M NaCl in several small protein peaks (peaks 2–4) and only these fractions were catalytically active (Fig. 4). Thus, it is obvious that peroxidase activity of rat IgGs is metal-dependent. Interestingly, according to the atomic emission data, all IgG fractions having affinity to Chelex (peaks 2–4, Fig. 4) contained all the metal ions that were found in the Ab preparations loaded on the Chelex (see above).

The catalysis mediated by artificial and natural Abzs is usually characterized by relatively low reaction rates: k_{cat} values for artificial and natural Abzs are approximately 10^2–10^6-fold lower than for the canonical enzymes with the catalytic activity of the respective type [1,4,5,7–14,25,33,34]. We have compared the relative specific peroxidase activity of our ten IgG preparations with that of a highly active preparation of HRP, the specific activity of which was ~10–50-fold higher than of
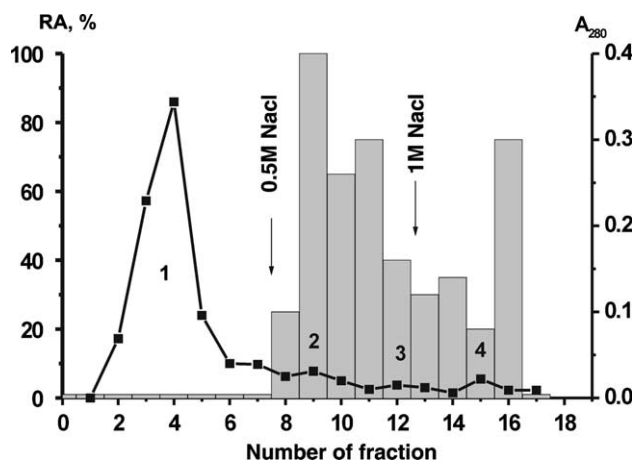
Fig. 4. Chromatography of rat IgGs on the Chelex column: (–), optical density at 280 nm; bars show the relative peroxidase activity (RA) of IgG fractions with different affinities for the chelating resin. RA of the fraction with the highest activity was taken for 100%.

commercially available HRP. The V_{max} and K_m values for HRP with DAB as substrate were estimated as 27.3 ± 8.0 µM/min and $1.0 \pm 0.25 \times 10^{-4}$ M ($k_{cat} = 10.8 \pm 2.5 \times 10^3$ min^{-1}; Fig. 5A).

We have estimated the values of $V_{max} = 60.0 \pm 8.0$ µM/min ($k_{cat} = 4.5 \pm 0.4 \times 10^2$ min^{-1}) and $K_m = 1.8 \pm 0.2 \times 10^{-4}$ M for DAB in the reaction catalyzed by IgGs (for example, Fig. 5B). The k_{cat} values for the different preparations of IgGs calculated using the total concentration of purified IgGs are summarized in Table 1. Interestingly, the relative affinities of 10 different IgGs to DAB were comparable ($K_m = 0.9$–2.2×10^{-4} M) with that for HRP. At the same time, the k_{cat} values (1.8–29.0×10^2 min^{-1}) varied significantly between the rats (Table 1). In contrast to known Abzs (see above), ten IgG preparations demonstrated high peroxidase activity (1.7–27%, taking the control HRP activity for 100%; Table 1).

Considering the data discussed above, one cannot exclude that the drastic differences in the peroxidase activity of Abs from individual rats may be a result of significant variations in the relative amount of different metal ions bound to these IgGs. In addition, the specific activity of ten IgGs was calculated using the total concentration of polyclonal IgGs. Since only a small fraction of IgGs can possess peroxidase like activity, the specific activity of the catalytic fraction of Abs may in fact be ~5–10-fold higher than that of the preparation analyzed (Table 1).

According to our preliminary data, all ten analyzed preparations of IgGs effectively oxidized not only DAB, but also other HRP substrates, toxic, mutagenic, and carcinogenic compounds such as o-phenylendiamine, phenol, p-hydroquinone, α-naphthol, etc. (to be reported elsewhere). Thus, it is possible that peroxidase IgGs in mammals can have a protective biological function similar to that of peroxidases in plants and in bacteria.

In addition to superoxide dismutase, catalase and glutathione peroxidase, IgG peroxidase can be considered as a very promising antioxidant of mammalian blood. As mentioned above, human sera contain Abs with superoxide dismutase activity. It seems likely that some Abzs can convert oxygen reducing it from $O_2^{\bullet-}$ into hydrogen peroxide [20,21], while

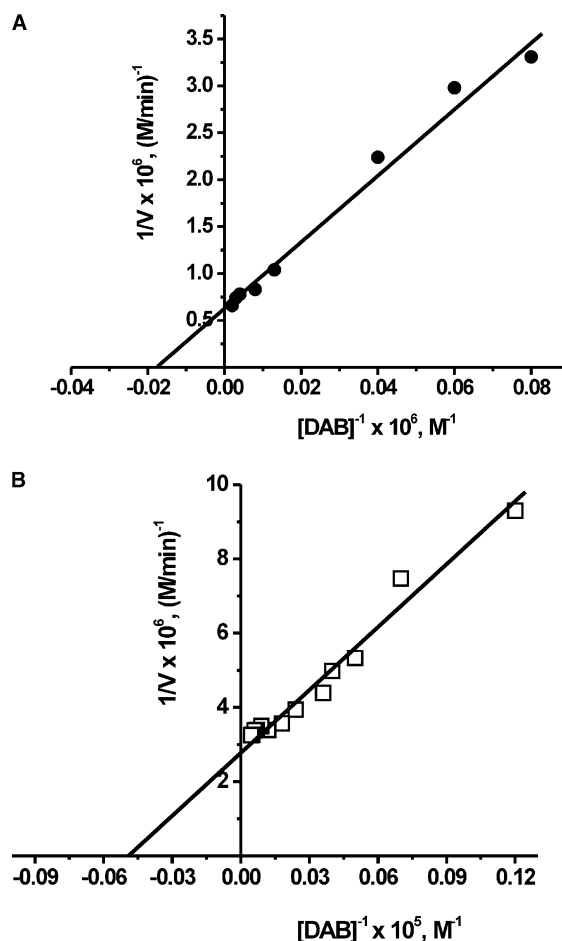Fig. 5. Determination of the K_m and V_{max} values for DAB in the reaction catalyzed by HRP (A) and IgG from the serum of one rat (B) using Lineweaver–Burk plot; 2.5 nM HRP and 130 nM IgGs were used.

Table 1
Variations in the relative specific peroxidase activity of IgGs from the sera of different rats

Number of IgG preparation	k_{cat} (min^{-1})	The relative activity (%)[b]
1	2.0×10^{3a}	18.5
2	6.2×10^2	5.7
3	3.3×10^2	3.1
4	8.3×10^2	7.7
5	1.8×10^2	1.7
6	2.9×10^3	27.0
7	6.2×10^2	5.6
8	1.0×10^3	9.3
9	4.6×10^2	4.3
10	2.1×10^2	1.9
HRP	10.8×10^3	100%

[a]The error was within 10–30%.
[b]The relative specific activity of HRP was taken as 100%.

other Abs with peroxidase activity neutralize H_2O_2. Taken together, these observations suggest that the specific repertoire of different polyclonal Abs can serve as an additional natural system of detoxication of different reactive oxygen species as well as oxidation of toxic, mutagenic, and carcinogenic compounds.

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