

Dual method for the determination of peroxidase-activity and total peroxides - iodide leads to a significant increase of peroxidase-activity in human sera

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Determination of peroxides and peroxidase-activity

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This work is dedicated to the late Prof. Marianne Hayn.

Abstract

Peroxidases are very important enzymes e.g. as preventive antioxidants by removing noxious peroxides from the blood. For this reason we evaluated a colorimetric method which detects the activity of endogenous peroxidases by their reaction with hydrogen peroxide, using tetramethylbenzidine as the chromogenic substrate. This assay design can be easily reversed by change of the variable compound to measure also total peroxides in plasma or serum.

An increased total antioxidant status was reported previously by the addition of iodide to human serum. In this study iodide activated the endogenous peroxidases significantly in comparison to control sera and isomolar NaCl as well as horse radish peroxidase. Corresponding to the increased peroxidase-activity a concomitant decrease of total peroxides occurred in the same samples.

This exchangeable assay design is a beneficial opportunity to screen total peroxide levels as well as peroxidase-activity in human sera without time consuming preparations. The method proved to be simple and is favourable due to its specificity, reproducibility and low costs. Moreover, we were able to find an explanation for the increased total antioxidant status in presence of iodide, which is presumably an indirect protective effect via an enhanced activity of enzymatic antioxidants, thereby reducing endogenous peroxides.

Key-words:

Iodine, oxidative stress, lipidperoxidation, peroxidases, peroxide, total antioxidant status, salt,

Introduction

3,5,3',5'-Tetramethylbenzidine (TMB) is an innocuous derivative of benzidine, which was used as a reagent for the detection of blood [1]. The oxidation of TMB by horseradish peroxidase (HRP)/H₂O₂ was reported by Josephy et al [2]. The advantage of this system is based on the very stable oxidation products of TMB at acid pH and that HRP is active over a wide pH range. Among the three characterized TMB oxidation products are the TMB cation free radical (one-electron oxidation product) which is in equilibrium with the charge-transfer complex and the diimine derivative of TMB (two-electron oxidation product). The charge-transfer complex is responsible for the blue colour in the course of TMB oxidation, while further oxidation yields the yellow 2-electron oxidation product (Fig.1).

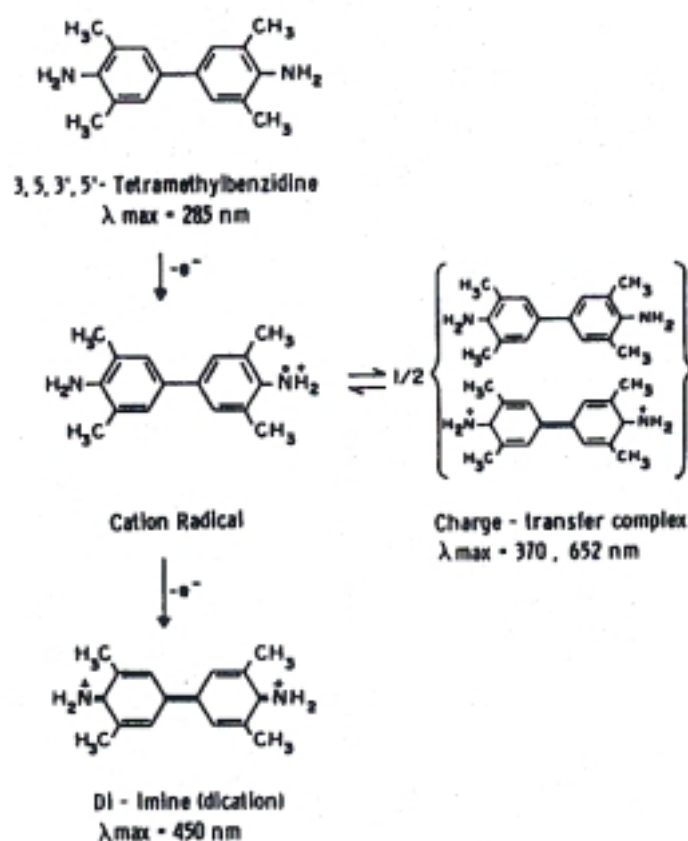
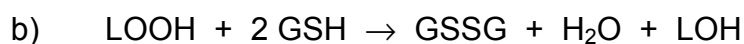


Fig. 1 Chemical structures of TMB and its oxidation states according to Josephy et al. [3]

Peroxidases are a family of wide-spread enzymes [4] which perform distinct tasks. On the one side they act as preventive antioxidants to detoxify damaging lipid

peroxides or other peroxides from blood and organic substrates. On the other side these enzymes function as starters for oxidative reactions, thereby generating a source for reactive oxygen species like HOCl or HOI. Such reactions are induced by activated neutrophils in the course of their antibacterial and antifungal actions [4]. Peroxidases can also modify LDL in the presence of H₂O₂ or lipid hydroperoxide (LOOH) [5]. Vice versa, a special type of peroxidases, the glutathione peroxidases (GPX), are very important as antioxidant defence enzymes, removing peroxides and H₂O₂ by coupling its reduction to H₂O or LOH with oxidation of reduced glutathione (GSH):



GPX enzymes are widely distributed in animal tissues and also in human blood (high in whole blood, low in plasma) [4]. Most of the GPXs are selenium-dependent, i.e. they consist of four protein units, each of which contains one atom Se at its active site.

In some studies on patients with degenerative diseases [6,7] an increase of the activities of plasma catalase and glutathione peroxidase was found in patients after drinking cures with an iodine brine. There is also much evidence for interactions between iodine/iodide and different peroxidase systems in vitro [8,9] as well as in vivo [4,10]. Some other studies measuring the total antioxidant status [11] have shown an antioxidative effect of iodide at concentrations of about 15 µM.

The present study presents a simple and quick method to measure peroxidase-activities on the one side and peroxide levels on the other side as a dual assay system. Furthermore, we tried to find out the influence of iodide, added to human serum in concentrations which could also be obtained with a local iodide brine by balneotherapeutic treatments, on peroxidase activity using this colorimetric assay. In addition the level of peroxides and the antioxidative capacity was determined in the same serum samples.

Materials and Methods

Patients and Blood Sampling

We enrolled 19 volunteers aged 30-65 years. Blood was drawn and centrifuged after 30 min. at 2000 x g for 10 min. Serum samples of all subjects were kept at -20°C until analysis (not later than 2 weeks).

Incubation procedure

Into an uncoated microtiter plate (96 wells, Nunc, Denmark)) 10µl serum and 10 µL of either NaI or NaCl (Sigma, USA) solution were added to the reaction mixture, to reach the final concentrations of 0,35 µM; 0,7 µM; 1,4 µM; 3 µM; 10 µM and 15µM of both halides in the test sample, respectively (total volume: 220 µL). In the case of controls, 10µl bidistilled water were added instead of NaCl and NaI. Each sample was tested in duplicate.

To determine significant differences paired t-test was used, $p < 0,05$ means significantly different.

Intra- and interassay coefficient of variation

Three fresh serum samples with distinct peroxidase-activity were used to validate the method. Five plates (96-multiwell) were used to measure each sample 20 times on each plate. Thus, 100 single determinations were available for statistical analysis for each serum.

To determine the recovery-rate an aliquot of 2,5 and 25 mU/ml HRP, respectively was added to each serum.

Peroxidase-activity assay

Determination of peroxidase-activity in serum was done by the the reaction of endogenous peroxidases with hydrogen peroxide, using TMB as the chromogenic substrate.

Assay protocol: 10µl standards (2,5 mU/ml and 25 mU/ml HRP) and samples were incubated with the reaction mixture (200 µl), consisting of hydrogen peroxide (30 %), TMB, and substrate buffer in a proportion of 1:10:100, in uncoated microtiter plates. Ingredients (TMB, substrate buffer and stop solution) were described elsewhere [12]. First absorbance reading was done immediately at a wavelength of 450 nm

(reference: 620 nm) in a plate reader. After an incubation period for 15 min. (\pm 5 min.) the reaction was finished by the addition of the stop solution and a second absorbance reading at 450 nm (reference: 620 nm). Serum peroxidase activity was calculated as the difference of the absorbance readings relating to the HRP standard curve. Results were expressed as mU/ml.

Total peroxide determination

Total peroxide levels in serum were measured by the same principle as described for the peroxidase-activity with one exception: Instead of peroxide, HRP was added to the reaction mixture, as described previously [13].

Assay protocol: 10 μ l standards (10 μ M – 1 mM H₂O₂) and samples were incubated with the reaction mixture (200 μ l), consisting of HRP (25 mU), TMB, and substrate buffer in a proportion of 1:10:100, in uncoated microtiter plates.

First absorbance reading was done immediately at a wavelength of 450 nm (reference: 620 nm) in a plate reader. After an incubation period for 15 min. (\pm 5 min.) the reaction was finished by the addition of the stop solution and a second absorbance reading at 450 nm (reference: 620 nm). Serum peroxide levels were calculated as the difference of the absorbance readings relating to the hydrogen peroxide standard curve. Results were expressed as "arbitrary units".

Total Antioxidant Status (TAS)

The TAS was measured using a colorimetric assay (Randox Laboratories, Crumlin, U.K.). The chromogen ABTS® (2,2'-Azino-di[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase and hydrogen peroxide to produce the ABTS radical cation. The ABTS radical is detectable due to its blue-green colour which is measured at 600 nm at 37°C. Antioxidants in the sample suppress the formation of the radical cation to a degree which is proportional to their concentration. Values are expressed as mmol/L.

Results ([Fig. 2-6 on one extra-page](#))

Validation of the total peroxidase-activity assay in serum

The peroxide/peroxidase kinetic follows a linear order as shown in [Figure 2](#) for peroxidase-activity and in [Figure 3](#) for peroxides.

We investigated 3 distinct sera with varying peroxidase-activities in the range of 15,18 –17,54 mU/ml. For the evaluation of the intra- and inter-assay variance these samples were measured 20 times on 5 different microtiter plates. Results are shown in Table 1.

From these results the intra-assay variance and inter-assay variance were calculated as 4,50 % indicated in Table 2. There were no significant differences between and within plates (t-test). Within one plate the coefficients range from 3,5 to 6 %, between plates from 3 to 6% for single sera.

Recovery rates were determined by the addition of HRP with an activity of 2,5 mU/ml and 25 mU/ml, respectively. Each serum was measured 10 times in duplicate.

To determine the recovery rate, a defined HRP (2,5 mU/ml and 25 mU/ml) was added to 3 sera in the range of 5-18 mU/ml (Table 3). The recovery rate was found in between 105,6 to 116,3 %.

The detection limit y_N results from [14] : $y_N = \hat{y}_L + 3s_B$ with \hat{y}_L as mean of 10 single determinations of blind value and s_B as its standard deviation and results in 0,579 mU/ml.

Addition of NaI/NaCl on peroxidase-activity

In this experimental series a highly significant increase of peroxidase activity was found by the addition of NaI compared to NaCl in the range between 0,35 – 0,7 μM .

NaI enhanced peroxidase-activity in comparison to control serum (without addition) as well beginning at concentrations of 0,35 μM ($p < 0,001$, [Figure 3](#)). In the opposite the addition of NaCl leads to a weak decrease in peroxidase-activity in comparison to control serum. NaI concentrations beginning with 1,4 μM could not be measured with our test system, because the reaction was accelerated so much that the optical density was out of range within a few seconds.

Effect of NaI/NaCl on peroxidase-standards:

To find out, if the increase of activity in the peroxidase-activity assay reflects really the activity of peroxidase, a series of peroxidase standards was tested with and without addition of NaI or NaCl, respectively.

As could be seen from Table 4, iodide enhances the peroxidase-activity also in HRP samples in a similar kind to endogenous peroxidases in serum compared to chloride. Buffer without HRP did not show any relevant signal in the peroxidase-activity assay.

Effect of NaCl/NaI on total peroxide concentrations in serum:

[Figure 4](#) shows the serum peroxide level of 19 samples with and without addition of NaCl and NaI. By the addition of increasing NaI concentrations ($> 1,4 \mu\text{M}$) peroxide levels decreased significantly even to a complete quench at $10\mu\text{M}$, i.e. peroxides are no longer measurable in such a manner due to activity of peroxidases into harmless metabolites. Chloride decreases the peroxide level as well, although less effectively, beginning with $3 \mu\text{M}$. The specific iodide effect was further supported by an increase of the total antioxidant status by the addition of NaI-solution in the range of $3\text{-}15 \mu\text{M}$ in comparison to isomolar NaCl-solution ($p=0,019$ t-test, [Fig. 5](#)).

Discussion

Recently, an increased total antioxidant status was reported in human sera in presence of iodide [11]. This is somewhat contradictory with respect to the possible pro-oxidative effects of halides and therefore we assumed, that an increased total antioxidant status might be an indirect effect of NaI. There are multiple connections between iodine or iodide (and other halides) with different peroxidases: thyroid peroxidase and also HRP [15] in their role for iodide oxidation and in consequence for thyroid hormone synthesis, the influence of iodide on the lactoperoxidase system as a natural antimicrobial system [16] and, as some authors suppose, a general antioxidant action of iodide together with peroxidases in all living organisms with iodine-concentrating cells from primitive algae to recent vertebrates [17,18].

The interlocking between iodine and peroxidase-activity became the hypothesis of this study i.e. iodine leads presumably to an increase of peroxidase-activity and therefore to an increased total antioxidant status. For that reason we evaluated a new mutual assay design for the determination of both peroxidase-activity and peroxide levels as well.

During several years benzidine was used for the detection of blood as well as in industry in the synthesis of dyes [19]. Due to the carcinogenic properties of this aromatic amine, the safer substitute TMB was described by *Holland and Saunders* [20]. This sensitive and specific agent resulted in negligible yield of tumours in comparison to benzidine when it was administrated to rats. Because of the fact that it is innocuous and sensitive it became the favourable chromogenic substrate in modern enzyme linked immuno sorbant assay's (ELISAs) [21]. In this study we describe a new application for this famous substrate with respect to its oxidation by HRP-H₂O₂. This type of reaction is the basic mechanism to detect both peroxidase-activity and peroxide levels in blood serum or plasma in this dual assay design. In case of enzyme-activity determination excess hydrogen peroxide was added, while HRP was added for the measurement of total peroxides. The new dual-assay has proved to be simple, cheap and reliable and is characterized by its linearity and accuracy with an intra- and interassay variance of 4.5%.

Only few numerical data are available on peroxidase activities in human blood or serum. Assuming that the predominant peroxidases are the antioxidatively effective glutathione peroxidases (GPX), its content was reported to be low in blood plasma,

but relatively high in erythrocytes and in whole blood [4]). On the other side, values of about 200 mU/ml total GPX were found in plasma of cure patients i.e. diabetics or patients with cardiovascular diseases by an enzymatic test [6,7]. But it must be kept in mind that also other peroxidases, e.g. myeloperoxidase (MPO) are present in abundance in neutrophils, monocytes and macrophages. MPO has been localized in macrophage-rich atherosclerotic lesions [5] and can be regarded as 'radical starter', as it produces hypochlorous acid and in consequence might oxidize lipoproteins. In case of arachidonic acid metabolite oxidation another group of peroxidases, the cyclooxygenases, are important as well.

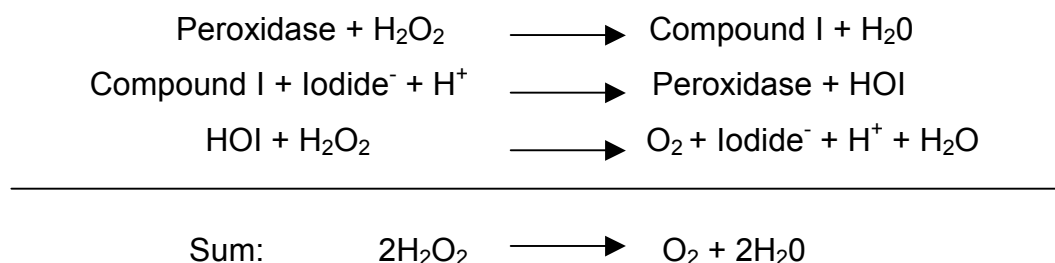
The previously reported increase in total antioxidant status in human serum according to NaI supplementation was confirmed in this study (see [Fig. 5](#)). The antioxidative potential of iodide became significant at a concentration of 3µM in comparison to controls (without salt supplementation) and NaCl, which remained at base line up to a concentration of 15 µM. The addition of 1,4 µM NaI resulted in a rise by leaps and bounds of the peroxidase-activity to an extent that the optical density was no longer measurable as indicated in [fig 3](#), while NaCl didn't effect peroxidase-activity at all. On the contrary we found a concomitant peroxide decrease at 1,4 µM NaI, which abolished the available peroxides almost at 3 µM. This is conclusive with respect to the enhanced total antioxidant status at the same concentration. Although NaCl didn't affect peroxidase-activity, a slow decrease of the peroxide level was observed even in presence of this salt. At a concentration of 15µM about one third of the initial peroxide level could be detected. This decrease is presumably caused by myeloperoxidase, which is released by activated neutrophils both into the phagocytic vacuole and the extracellular environment by degranulation. MPO initially reacts with equimolar concentration of H₂O₂ to form the short-lived redox intermediate compound I [22]. Compound I is very reactive in oxidizing chloride to liberate HOCl and regenerating the native enzyme (equations 1 and 2) [23,24].



The abolishment of peroxides either by increased peroxidase-activity or through metabolism to HOCl by MPO is further evidence for the substrate specificity in the peroxide test.

In conclusion have evaluated a new dual test for the measurement of peroxidase-activity on the one hand and peroxide levels on the other hand. These tests are suitable as screening tests with respect to their simple handling, reliability and low costs. Moreover these tests are characterized by their linearity, good precision and due to an endpoint determination.

Together with the result of an increased total antioxidant status through the addition of iodide at μ molar concentrations, our present data plead for an indirect antioxidant effect of iodide via peroxidases, thereby reducing total peroxides in the sample. It was shown [25,26,27] that iodide converts peroxidases into effective catalases via the reactions:



Thus, thyroid peroxidase, lactoperoxidase and horseradish peroxidase (and the same can be anticipated for other peroxidases) display catalatic activity in the presence of iodide.

The hypoiodous acid reacts also with excess iodide to form I_2 and in a further reaction to form triiodide. These different peroxidase-catalyzed reactions are coordinated by the so-called "Iodine clock" [27]. The catalytic activity is totally dependent on iodide. It is abolished in the presence of iodide acceptors and is inhibited at high concentrations of iodide [25].

The results are also consistent with previous findings [6,7], who demonstrated a specific increase of plasma GPX (and partially of catalase) in patients after drinking cures with an iodine brine.

According to *Krizek et al.* [28], iodine levels in the serum might increase to about 3 μM after drinking an iodine brine, which is exactly the beneficial concentration range. Controlled studies including further parameters of the pro/antioxidants with cure patients are needed to assess the real significance of this iodine effect.

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Legends

Figure 1: Chemical structures of TMB and its oxidation states according to [3]

Figure 2: Standardization of peroxidase-activity assay

Figure 3: Standard curve for the determination of total peroxides

Figure 4: Peroxidase activity of blood serum (n = 19) in the peroxidase-activity assay in presence of NaI and NaCl in the range between 0,35 and 15 μ M. Means \pm SD.

*** p < 0,001 vs isomolar NaCl

Figure 5: Peroxides of blood serum (n = 19) in the peroxide assay in presence of NaI and NaCl in the range between 0,35 ... 15 μ M. Means \pm SD.

Figure 6: TAS of blood serum (n = 19) in the TAS assay. Means \pm SD.

*** p < 0,001

** p < 0,02

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Table 1: Peroxidase activity of 3 distinct sera on 5 different plates. Unless otherwise stated, the results are expressed as mean values \pm standard deviation (SD).

	Peroxidase-activity [mU/ml] \pm SD		
	serum 1	serum 2	serum 3
Plate 1	5,12 \pm 0,48	9,26 \pm 0,49	17,90 \pm 0,59
Plate 2	4,89 \pm 0,30	9,00 \pm 0,31	17,81 \pm 0,62
Plate 3	5,09 \pm 0,27	8,61 \pm 0,50	17,61 \pm 0,59
Plate 4	5,07 \pm 0,21	8,47 \pm 0,35	18,06 \pm 0,42
Plate 5	5,71 \pm 0,28	9,32 \pm 0,33	16,34 \pm 0,47

Table 2: Coefficients of variation within and between plates

Coefficients of variation (CV) within and between plates [%]				
	serum 1	Serum 2	serum 3	Mean of CV within plates
CV plate 1	9,30	5,33	3,32	5,98
CV plate 2	6,18	3,47	3,49	4,38
CV plate 3	5,23	5,85	3,36	4,83
CV plate 4	4,16	4,19	2,32	3,56
CV plate 5	4,83	3,56	2,89	3,76
Mean of CV between plates	5,95	4,48	3,08	4,50

Table 3: Recovery rate of peroxidase-activity of HRP

	serum 1		Serum 2		serum 3	
	Recovery		Recovery		recovery	
n	[mU/ml]	[%]	[mU/ml]	[%]	[mU/ml]	[%]
10	5,46	105,6	10,39	116,3	18,90	107,8

Table 4: Effect of NaI/NaCl on peroxidase standards (ST 1, ST 2)

Additive	Peroxidase activity [mU/ml]			
	NaCl	NaI	NaCl	NaI
Buffer	Not detectable			
Addition	Standard 1 [2,5 mU/ml]		Standard 2 [25m U/ml]	
Without	10,26		24,90	
0,35 μ M	9,77	10,41	24,69	25,26
0,70 μ M	10,02	10,66	24,69	25,58
1,4 μ M	9,49	10,99	24,78	25,91
3,0 μ M	9,76	n.d.	24,68	n.d.
10 μ M	9,93	n.d.	24,70	n.d.
15 μ M	9,96	n.d.	24,71	n.d.