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PROCEDURE FOR BLOOD GLUTATHIONE PEROXIDASE DETERMINATION IN CATTLE AND SWINE

By

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AGERGAARD, N. and P. THODE JENSEN: *Procedure for blood glutathione peroxidase determination in cattle and swine.* Acta vet. scand. 1982, 23, 515—527. — An improved testing system has been developed for direct measurement of glutathione peroxidase activity in heparinized whole blood at 37°C. Without loss in net yield the consumption of reagents has been found to be considerably lower with the new technique than with previously described techniques. Within the range 0—700 mKat/l the GSH-Px activity in red cells may be measured with a high degree of accuracy and reproducibility without preceding separation and washing. The stability of the enzyme in bovine and porcine whole blood at 22°C, 4°C, and —20°C was determined.

glutathione peroxidase; selenium status; methodology; storage; cattle; swine.

On account of the regularly observed correlation between glutathione peroxidase GSH-Px (EC 1.11.1.9) activity and selenium status (cf. *Underwood* 1977, *Van Vleet* 1980, *Sunde & Hoekstra* 1980) the determination of GSH-Px levels in blood and tissue has been adapted as a useful procedure for detecting selenium deficiency.

Most of the hitherto described methods for GSH-Px determination in domestic animals involve laborious separation and washing procedures. The present work was carried out in order to optimize the determination of GSH-Px activity directly on heparinized whole blood.

Through systematic adjustment of each of the component reactions the technique has been optimized at 37°C in respect

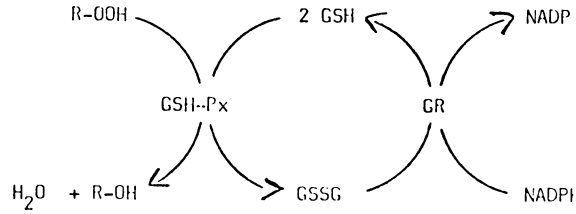
of the concentration of donor and receptor substrates, ionic strength, and pH.

Moreover, the GSH-Px stability in bovine and porcine whole blood has been determined at different temperatures.

MATERIAL AND METHODS

Heparinized blood samples (143 USP units of Sodium-heparinate/10 ml, Venoject® tubes) have been used for examination of the GSH-Px reaction kinetics. To examine the stability of the enzyme, samples of whole blood have been stored at 22°C, 4°C and —20°C.

The enzyme activity determination is based upon measurement of the rate of glutathione [GSH] oxidation by tert. butyl hydroperoxide [TBH] = [R-OOH] as catalyzed by the GSH-Px present in a hemolysate or plasma sample (*Günzler et al.* 1974). Because of the coupled test system there will be no progressive loss of GSH, its concentration being maintained at a constant level by the addition of exogenous glutathione reductase [GR] and NADPH, which will immediately convert any oxidiced glutathione [GSSG] produced to the reduced form GSH:



The rate of GSH formation is monitored through the NADPH consumption as recorded at 366 nm. Two minutes after the reaction is started by the addition of TBH, the NADPH consumption rate is recorded over a period of 60 s. The difference in consumption rate between sample and blank determines the GSH-Px activity.

Hemolysates were prepared by making a 21 × dilution of whole blood in Drabkin's reagent (KCN 0.4 mmol/l, $K_3Fe(CN)_3$

Reduced glutathione [GSH], glutathione reductase [GR] from yeast (120 U/mg) and NADPH (highest purity) were purchased from Boehringer. Tert. butyl hydroperoxide [TBH] (80 %) was from Merck-Schuchardt and bovine hemoglobin from Sigma. All other reagents were products "pro analysi" from Merck-Darmstadt.

0.3 mmol/l and $NaHCO_3$ 6.0 mmol/l) using an automatic diluter/dispenser (Gilford). The GR reagent was used as a freshly prepared dilution. The GSH and NADPH solutions were prepared once a week and stored at —20°C, while the TBH was prepared every second week and stored at 4°C. Redistilled water was used for all preparations.

Because of the fact that hemoglobin possesses peroxidative activity, its interference with the GSH-Px reaction was studied using purified bovine hemoglobin.

The composition of the reagents and the concentrations in which they are used are shown in Table 1, together with the complete assay procedure.

Table 1. Procedure, and composition of reagents, for photometric assay of GSH-Px.

Reagent	Volume ml	Final concentration
Potassium phosphate buffer		100 mmol/l
400 mmol/l, pH 7.2	0.5	
EDTA, 16 mmol/l		4 mmol/l
GSH, 60 mmol/l	0.2	6 mmol/l
Glutathione reductase, 15 U/ml	0.05	0.375 U/ml
Hemolysate	0.02	
Redestilled water	0.48	
10 min preincubation at 37°C		
NADPH, 3 mmol/l in 0.1 % NaHCO	0.2	0.3 mmol/l
Tert. butyl-hydroperoxide, 6.3 nmol/l	0.5	1.575 mmol/l
Start of reaction		
Recording of NADPH consumption (366 nm)		

The K_m constants for the substrates were determined by the Lineweaver-Burk method (*Lineweaver & Burk* 1934).

The test was performed at 37°C ± 0.05°C using a Gilford spectrophotometer with a computer/printer unit. In accordance with the SI nomenclature (*Moss* 1978) and the experimental conditions given above, enzyme units for GSH-Px activity were calculated as follows:

$$\Delta A_{366}/\text{min} \times 5303 = \text{mKat/l (whole blood)}$$

using a molar extinction coefficient for NADPH of $3.3 \times 10^3 \times 1 \times \text{mol}^{-1} \times \text{cm}^{-1}$.

Duplicate determinations on bovine and porcine samples were used for calculation of the standard deviation. The calculations was made by means of the formula $s = \sqrt{\Sigma d^2 \times 2n^{-1}}$, where d is the difference between duplicate determinations and n their number. A reserve of pooled and freeze-dried blood samples from cattle was kept for up to 6 months. These samples were used for determination of between-assay variations.

RESULTS

The effect of pH on the GSH-Px reaction rate is shown in Table 2. In the pH range 6.2—8.2 there is a steady rise in the reaction rate, amounting to an increase in ΔA/min of 0.16 for both the sample and the blank reaction. The net yield is maximal and relatively constant over the pH range 6.95—7.70. The increase of the total reaction at pH > 7.7 is caused entirely by an increase of the blank reaction. It appears from the table that the highest yield is obtained at pH 7.2.

Table 2. The influence of pH upon the relative net activity of GSH-Px in bovine and porcine blood.

	Cattle (n = 8)			Swine (n = 8)		
	ΔA/min		relative net	ΔA/min		relative net
pH	blank	total*	activity	blank	total*	activity
6.20	0.014	0.049	0.56	0.004	0.055	0.89
6.70	0.026	0.073	0.76	0.026	0.078	0.91
6.95	0.037	0.091	0.87	0.047	0.101	0.95
7.20	0.057	0.119	1.00	0.083	0.140	1.00
7.45	0.088	0.152	1.02	0.112	0.168	0.98
7.70	0.119	0.178	0.95	0.143	0.195	0.91
8.20	0.178	0.205	0.45	0.208	0.227	0.33

* Range of s.e.: Cattle 0.002—0.008; Swine 0.002—0.006.

Table 3 shows the effect of ionic strength (μ) on the enzyme activity at pH 7.2. Maximal GSH-Px activity is obtained at $\mu = 0.05$. Unlike the net yield, the blank reaction increases proportionally with the ionic strength.

The temperature effect on the GSH-Px activity is shown in Table 4. The mean temperature coefficients obtanied over the range from 25°C to 42°C were higher in porcine ($Q_{10} \sim 3.0$) than in bovine blood ($Q_{10} \sim 1.7$).

Table 3. The influence of ionic strength (μ) on the relative net activity of GSH-Px at pH 7.2

Ionic strength at pH 7.2	Cattle (n = 8) ΔA/min blank	Cattle (n = 8) ΔA/min total*	Cattle (n = 8) relative net activity	Swine (n = 8) ΔA/min blank	Swine (n = 8) ΔA/min total*	Swine (n = 8) relative net activity
0.00625	0.011	0.047	0.51	—	—	—
0.0125	0.018	0.067	0.69	0.015	0.051	0.76
0.025	0.033	0.103	0.99	0.022	0.065	0.91
0.05	0.041	0.112	1.00	0.031	0.078	1.00
0.1	0.050	0.120	0.99	0.036	0.083	1.00
0.2	0.057	0.119	0.87	0.038	0.083	0.95
0.4	0.065	0.116	0.72	0.040	0.084	0.93
0.8	0.077	0.124	0.66	0.050	0.086	0.76

* Range of s.e.: Cattle 0.004—0.018; Swine 0.005—0.012.

Table 4. Relative net activity of GSH-Px in bovine and porcine blood at temperatures from 25°C to 42°C.

Temperature °C	Cattle (n = 5) ΔA/min blank	Cattle (n = 5) ΔA/min total*	Cattle (n = 5) relative net activity	Swine (n = 5) ΔA/min blank	Swine (n = 5) ΔA/min total*	Swine (n = 5) relative net activity
25	0.021	0.068	1.00	0.019	0.036	1.00
30	0.034	0.097	1.34	0.029	0.054	1.47
37	0.064	0.163	2.11	0.056	0.110	3.18
42	0.097	0.232	2.87	0.078	0.165	5.11

* Range of s.e.: Cattle 0.006—0.026; Swine 0.003—0.014.

Fig. 1 shows the reaction rates for a high-activity and a low-activity hemolysate, and the blank reaction. It appears that the reactions are stabilized within 90 s after start, and that the curves thereafter remain linear for at least 2 min. Hemolysates with an activity exceeding 700 mKat/l were re-analysed after a suitable further dilution with 0.9 % NaCl.

The effect on the total reaction rate of varying the substrate concentration is shown in Fig. 2, together with the blank reaction rate. The rates for all substrate concentrations were determined simultaneously. In the absence of TBH the enzymatic reaction rate was always zero, whereas omission of GSH never resulted in an enzymatic activity of less than $\Delta A/min = 0.030$, indicating the presence of small amounts of endogenous GSH in the hemolysate.

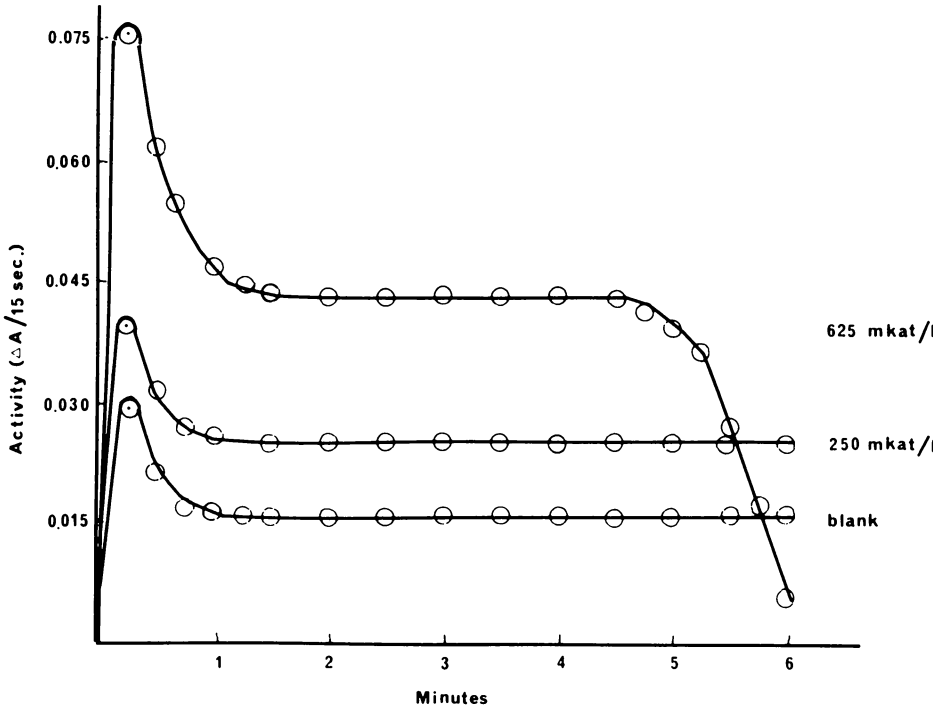


Figure 1. Curves showing the GSH-Px reaction for a high-activity and a low-activity hemolysate, compared with the blank reaction curve.

The peroxidase activity resulting from addition of purified bovine hemoglobin to the Drabkin solution to a concentration of 75 mg/l in the final reaction mixture was found to be insignificant ($\Delta A/min < 0.005$). This hemoglobin concentration equals the maximal concentration occurring in the reaction mixture when the analysis is performed on whole blood. Under the conditions optimized with respect to the net yield of the GSH-Px reaction, K_m values of 1.15 mmol/l and 0.59 mmol/l were obtained with cattle blood for GSH and TBH, respectively. With swine blood the K_m values were 2.01 mmol/l and 0.69 mmol/l, respectively.

Table 5 shows the influence of storing conditions on the GSH-Px activity in cattle and swine whole blood. Three series of identical samples were stored at respectively, 22°C, 4°C, and —20°C. The samples were selected in such a way that they would represent a reasonable range of activity. During the first day

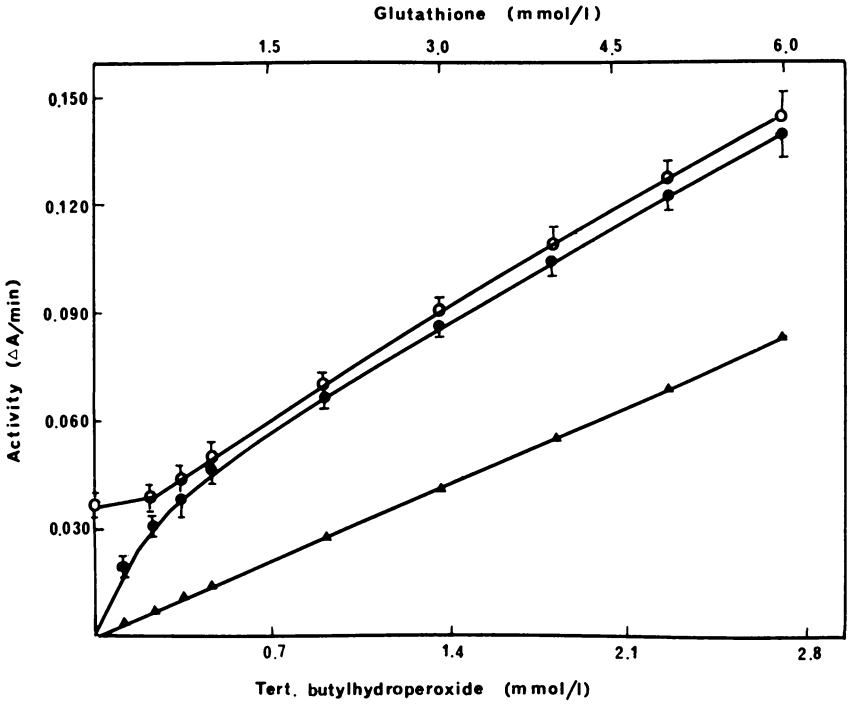


Figure 2. GSH-Px activity as a function of substrate concentration. For each plot the mean standard error for 8 determinations is indicated. The values for GSH (○——○) were obtained with a TBH concentration of 1.6×10^{-3} mol/l. Those for TBH (●——●) were obtained with a GSH concentration of 6.0×10^{-3} mol/l. The blank reaction (▲——▲) is the same for both sets of conditions.

Table 5. The influence of storing conditions upon the GSH-Px activity in whole blood from 8 cattle with initial activities ranging from 91 to 365 mKat/l and from 8 pigs with initial activities ranging from 252 to 321 mKat/l. Figures indicate mean relative changes in activity

	22°C		4°C		−20°C	
Storage time (days)	Cattle	Pig	Cattle	Pig	Cattle	Pig
0	1.00	1.00	1.00	1.00	1.00	1.00
1	1.30	1.33	1.31	1.22	1.31	1.21
2	1.08	1.00	1.10	0.98	1.25	0.98
4	1.10	0.98	1.06	0.95	1.19	1.07
8	1.06	1.01	0.92	0.91	1.14	1.05
14					1.13	1.08
21					1.11	1.07
28					1.03	1.07

of storage the activity increased in both cattle and swine blood. After 2 days the activity in swine blood was back to roughly the initial level, whereafter it remained stable for at least 1 week at 22°C and 4°C, and for 1 month at —20°C.

The GSH-Px activity of hemolysates from frozen blood fell during storage, contrary to what was the case with the thawed whole blood, where the enzyme would keep for at least 6 h. In hemolysates derived from fresh blood the GSH-Px enzyme was found just as stable as in whole blood.

The intra-assay coefficient of variation was 2.3 % for 31 duplicate samples with activities ranging from 116 to 706 mKat/l. The between-assay coefficient of variation was 7.5 %, as determined during a 6-month period on 20 samples of a freeze-dried pool of blood with a mean activity of 226 mKat/l. The mean activity of the freeze-dried samples remained unchanged through the period of storing.

DISCUSSION

The increasing awareness of the nutritional importance of selenium has provided a marked stimulus for studies on the biochemical role of that metalloid. For instance, selenium is required for the formation of the GSH-Px enzyme in mammals (*Flohé et al.* 1973, *Rotruck et al.* 1973, *Oh et al.* 1974, *Allen et al.* 1975, *Anderson et al.* 1978, *Chavez* 1979 a & b) and avians (*Noguchi et al.* 1973, *Omaye & Tappel* 1974) and the bacterial enzymes formate dehydrogenase (E.C. 1.2.1.43) and glycin reductase (E.C. 1.4.2.1) (*Stadtman* 1980). From a physiological point of view it is of interest that the hitherto described selenium-dependent enzymes all belong to the group of oxido-reductases.

It was early recognized that in mammals GSH-Px is chiefly responsible for the protection of cell membranes from damage caused by organic peroxides. The most widely recognized function of the GSH-Px enzyme is to reduce peroxides (*Scott et al.* 1974, *Combs et al.* 1975, *Hoekstra* 1975). Various lipid peroxides, steroid peroxides, nucleic acid peroxides, prostaglandin peroxides and other organic peroxides, together with H_2O_2, have been shown to be acceptor substrate for GSH-Px (*Little & O'Brien* 1968, *Günzler et al.* 1972, *Little* 1972). Because almost all peroxidase substrates will elicit similar maximal velocities, similar to that elicited by H_2O_2, an acceptor-enzyme complex is most likely

not formed during catalysis. Unlike other peroxidases GSH-Px has a high specificity for its donor substrate, GSH, which is the only reductant of physiological importance (*Günzler et al.* 1972). No other thiol substrate studied so far has more than 30 %, and most of them less than 10 %, of the activity of GSH (*Flohé et al.* 1971). A detailed description of the reactions involved has recently been given by *Sunde & Hoekstra* (1980).

As stated under Results, a series of parameters of the GSH-Px system have been examined at 37 °C using heparinized bovine and porcine whole blood as sources of enzyme.

The coefficient of variation remained practically unchanged for each of the component reactions. Thus, the reproducibility is practically the same over a wide range of assay conditions. On the other hand, there is a great difference in reagent consumption and net yield. Thus, rise in pH and ionic strength caused a drastic increase of NADPH consumption. The increased consumption was mainly connected with the blank reaction, which increased proportionally to both pH and ionic strength.

At the optimal pH and ionic strength for GSH-Px determination (pH = 7.2, μ = 0.05) there is a relatively strong blank reaction. This must be accepted, even though it involves a reagent consumption equivalent to half the total amount necessary for the analysis. At 37 °C the blank reactions were about 3 times stronger than at 25 °C, but at the same time the net yield was more than doubled. Not only because of this increase should the determination of GSH-Px in blood and tissue be carried out at body temperature, but also because the experimental conditions ought to be kept as close as possible to normal physiological conditions.

The K_m value for GSH found with porcine whole blood in this study is comparable to the value determined by *Little et al.* (1970) in an experiment with highly purified GSH-Px from porcine blood. K_m determination is to some extent approximative, since the range of substrate concentrations is necessarily limited, both because of denaturation of the enzyme at higher TBH concentrations and because of the excessive non-enzymatic reaction rates at higher levels of GSH.

The concentration of GSH was limited to maximum 6 mmol/l (Table 1) to avoid product inhibition of glutathione reductase (*Flohé & Brand* 1970), the frequently used substrate concentration of $10 \times K_m$ in enzyme reactions having been found in-

expedient in this system, in which $[GSH]_0$ is kept at a constant level by means of the coupled GR-NADPH system.

Regardless of storing temperature and animal species (Table 5) an initial rise in GSH-Px activity occurred after sampling. In samples kept at 22°C or 4°C the activity was back to almost the initial level after 2 days, whereas in samples kept at —20°C this was not the case till after about 2 weeks. Therefore, if it is not possible to analyse the blood shortly after sampling, the influence of the storing conditions has to be considered.

In a recent paper, *Hussein & Jones* (1981) reported that in blod from cows, goats, and horses there was no change in GSH-Px activity after storage at —20°C, while this activity was greatly affected by storage at room temperature. They found a significant decrease in activity after 4 days at 20°C. Their results disagree with those of the present investigation, which indicate that except for the initial rise during the first 24 h practically no change in GSH-Px activity will occur even after 8 days at 22°C (Table 5). An explanation of the difference may be that *Hussein & Jones* have assumed that it was possible to use frozen samples for reference. However, as appears from this study, only fresh sample activities can be used as reference values for studying the effect of storing conditions.

For assaying the GSH-Px activity in thawed blood samples, hemolysates should be prepared immediately before the analysis. The reduced stability of GSH-Px in hemolysates prepared from frozen samples can possibly be explained by the observation (*Kraus & Ganther* 1980) that in GSH-Px glutathione is bound to selenium by a selenyl-sulfide linkage, which is apt to be cleaved by cyanide. *Kraus & Ganther* have demonstrated that cyanide sensitivity is correlated with the presence of an oxidized glutathione-enzyme moiety. Oxidized glutathione accumulates in red cells on storage, causing an increase of the oxidized, cyanide-sensitive fraction of GSH-Px, and an equivalent decrease of the reduced, cyanide-insensitive fraction (*Prohaska et al.* 1977). A similar phenomenon is known to occur in the case of red cell adenosine deaminase, where *Spencer et al.* (1968) have demonstrated that exchange reactions are taking place between reactive sulphydryl groups and oxidized glutathione, causing a changed isoenzyme pattern in which the oxidized fractions increase proportionally with storage time.

With the equipment and the procedure outlined above it is possible in a single run to measure GSH-Px activity over the range 0—700 mKat/l, which covers most levels met with in blood samples from cattle and swine.

REFERENCES

Allen, W. M., W. H. Parr, P. H. Anderson, S. Berrett, R. Bradley & D. S. P. Patterson: Selenium and the activity of glutathione peroxidase in bovine erythrocytes. Vet. Rec. 1975, *102*, 360—361.

Anderson, P. H., S. Berrett & D. S. P. Patterson: Glutathione peroxidase activity in erythrocytes and muscle of cattle and sheep and its relationship to selenium. J. comp. Path. 1978, *88*, 181—189.

Chavez, E. R.: Effect of dietary selenium on glutathione peroxidase activity in piglets. Canad. J. Anim. Sci. 1979a, *59*, 67—75.

Chavez, E. R.: Effect of dietary selenium depletion and repletion on plasma glutathione peroxidase activity and selenium concentration in blood and body tissue of growing pigs. Canad. J. Anim. Sci. 1979b, *59*, 761—771.

Combs, G. F., T. Noguchi & M. L. Scott: Mechanisms of action of selenium and vitamin E in protection of biological membranes. Fed. Proc. 1975, *34*, 2090—2095.

Flohé, L. & I. Brand: Some hints to avoid pitfalls in quantitative determination of glutathione peroxidase (E.C. 1.11.1.9). Z. Klin. Chem. Klin. Biochem. 1970, *8*, 156—161.

Flohé, L., W. Günzler, G. Jung, E. Schaich & F. Schneider: Glutathion-Peroxidase. II. Substratspezifität und Hemmbarkeit durch Substratanaloge. (Glutathione peroxidase. II. Substrate specificity and inhibition by substrate analogues). Hoppe-Seylers Z. Physiol. Chem. 1971, *352*, 159—169.

Flohé, L., W. A. Günzler & H. H. Schock: Glutathione peroxidase. A selenoenzyme. FEBS Letters 1973, *32*, 132—134.

Günzler, W. A., H. Kremers & L. Flohé: An improved coupled test procedure for glutathione peroxidase (E.C. 1.11.1.9) in blood. Z. Klin. Chem. Klin. Biochem. 1974, *12*, 444—448.

Günzler, W. A., M. Vergin, I. Müller & L. Flohé: Glutathion-Peroxidase. VI. Die Reaktion der Glutathion-Peroxidase mit verschiedenen Hydroperoxiden. (Glutathione peroxidase. VI. The reaction of glutathione peroxidase with different hydroperoxides). Hoppe-Seylers Z. Physiol. Chem. 1972, *353*, 1001—1004.

Hoekstra, W. G.: Biochemical function of selenium and its relation to vitamin E. Fed. Proc. 1975, *34*, 2083—2089.

Hussein, K. S. M. & B. E. V. Jones: Effects of different anticoagulants on the determination of erythrocyte glutathione peroxidase. Acta vet. scand. 1981, *22*, 472—479.

Kraus, R. J. & H. E. Ganther: Reaction of cyanide with glutathione peroxidase. Biochem. Biophys. Res. Commun. 1980, *96*, 1116—1122.

Lineweaver, H. & D. Burk: The determination of enzyme dissociation constant. J. Amer. chem. Soc. 1934, *56*, 658—666.

Little, C.: Steroid hydroperoxides as substrates for glutathione peroxidase. Biochem. Biophys. Acta 1972, *284*, 375—381.

Little, C. & P. J. O'Brien: An intracellular GSH-peroxidase with a lipid peroxidase substrate. Biochem. Biophys. Res. Commun. 1968, *31*, 145—150.

Little, C., O. Olinescu, K. G. Reid & P. J. O'Brien: Properties and regulation of glutathione peroxidase. J. Biol. Chem. 1970, *245*, 3632—3636.

Moss, D. W.: Nomenclature and units in enzymology. In: Principles of Enzymatic Analysis. Ed. H. U. Bergmeyer. Verlag Chemi 1978.

Noguchi, T., A. H. Cantor & M. L. Scott: Mode of action of selenium and vitamin E in prevention of exudative diathesis in chicks. J. Nutr. 1973, *103*, 1502—1511.

Oh, S. H., H. E. Ganther & W. G. Hoekstra: Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochemistry 1974, *13*, 1825—1829.

Omaye, S. T. & A. Tappel: Effect of dietary selenium on glutathione peroxidase in the chick. J. Nutr. 1974, *104*, 747—753.

Prohaska, J. R., S. H. Oh, W. G. Hoekstra & H. E. Ganther: Glutathione peroxidase: Inhibition by cyanide and release of selenium. Biochem. Biophys. Res. Commun. 1977, *74*, 64—71.

Rotruck, J. T., A. L. Pope, H. E. Ganther, A. B. Swanson, D. G. Hafeman & W. G. Hoekstra: Selenium: Biochemical role as a component of glutathione peroxidase. Science 1973, *179*, 588—590.

Scott, M. L., T. Noguchi & G. F. Combs: New evidence concerning mechanisms of action of vitamin E and selenium. Vitam. Horm. 1974, *32*, 429—443.

Spencer, N., D. A. Hopkinson & H. Harris: Adenosine deaminase polymorphism in man. Ann. Hum. Genet. 1968, *32*, 9—14.

Stadtman, T. C.: Selenium-Dependent enzymes. Ann. Rev. Biochem. 1980, *49*, 93—110.

Sunde, R. A. & W. G. Hoekstra: Structure, synthesis and function of glutathione peroxidase. Nutr. Rev. 1980, *38*, 265—273.

Underwood, E. J.: Trace Elements in Human and Animal Nutrition. 4. ed. Academic Press, New York 1977.

Van Vleet, J. F.: Current knowledge of selenium-vitamin E deficiency in domestic animals. J. Amer. vet. med. Ass. 1980, *176*, 321—325.

SAMMENDRAG

Analyseprocedure til bestemmelse af glutathion peroxidase i kvæg- og svineblod.

En analysemetode til bestemmelse af glutathion peroxidase i heparinstabiliseret fuldblod fra kvæg og svin er optimeret med hensyn til pH, ionstyrke og donor- og acceptorsubstrat-koncentrationer ved 37°C. I forhold til tidligere beskrevet teknik er reagensforbruget reduceret uden fald i nettoudbyttet. I intervallet 0—700 mKat/l måles GSH-Px aktiviteten i erytrocyter uden forudgående separations- og vaskeprocedurer. Enzymets stabilitet i fuldblod fra kvæg og svin er fastlagt ved 22°C, 4°C og —20°C.

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