

# Selenium and glutathione peroxidase in seminal plasma of men and bulls\*

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**Summary.** High levels of selenium and glutathione peroxidase (GSH-Px) were found in bull seminal plasma but low concentrations in human seminal plasma. In man the seminal plasma selenium was associated with two macromolecules separable by gel filtration, but no GSH-Px was found in the same fractions. Selenium in bull seminal plasma was associated with two proteins, which could be separated by gel filtration and anion exchange chromatography. Both macromolecules coeluted with GSH-Px activity and had identical optima at pH 7.0. Their responses to thermal treatment, however, differed. Seminal vesicle secretory fluid in the bull contained both these proteins, while the larger molecule was also found in fractionations of ampulla, prostate and Cowper's glands. The larger enzyme form is evidently a tetramer of the smaller one. Both enzyme forms were extremely sensitive to heavy metals and some divalent metal ions. GSH caused an activation while other reducing agents were suppressive. Triton X-100 had no effect, while sodium deoxycholate was inhibitory. These properties are typical for a phospholipid hydroperoxide GSH-Px. It is concluded that this selenium-dependent enzyme may be important in the protection of bovine spermatozoa against damage caused by oxygen radicals, while in man such a mechanism is not functional.

**Keywords:** selenium; glutathione peroxidase; seminal plasma; human; bull

## Introduction

The first evidence for selenium as an integral component of glutathione peroxidase (GSH-Px; EC 1.11.1.9) was obtained with the enzyme from rat erythrocytes (Rotruck *et al.*, 1973). In the same study the role of the enzyme in the protection of membrane lipids from oxidative damage was appreciated. GSH-Px activity has been found in the semen of several species including ram, dog, human, goat (Li, 1975) and bull (Brown *et al.*, 1977). In the bull GSH-Px is associated with the seminal plasma and not spermatozoa (Brown *et al.*, 1977; Brown & Senger, 1977; Smith *et al.*, 1979). Selenium, however, is found also in the spermatozoa, where it is presumably confined to structural macromolecules (Calvin & Cooper, 1979; Pallini & Bacci, 1979; Niemi *et al.*, 1981). The incorporation of <sup>75</sup>Se into bull seminal plasma takes place much faster than into the spermatozoa (Smith *et al.*, 1979) and selenium administration is rapidly reflected as an increase of the seminal plasma GSH-Px (Bartle *et al.*, 1980).

In bulls the seminal plasma selenium values are clearly higher than those in serum or blood (Smith *et al.*, 1979; Bartle *et al.*, 1980). Our own studies (Saaranen *et al.*, 1986) have also shown that the selenium concentrations are about 10 times higher in bull seminal plasma than in serum, while in man the seminal plasma selenium levels are low and less than half of those in serum. Moreover, the selenium concentrations in bull seminal vesicle secretion were comparable to those in the

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seminal plasma, but the tissue levels in testis and epididymis exceeded those in the seminal vesicle or other accessory sex glands. In man the tissue concentrations of selenium were comparable to those of the bull.

The aims of the present study were to examine (1) the association of selenium and GSH-Px with macromolecules in seminal plasma of men and bulls and (2) the role of different reproductive organs in the secretion of GSH-Px and selenium.

## Materials and Methods

**Chemicals.** The reagents utilized in the electrothermal atomic absorption spectrophotometry (ET-AAS) were as specified previously (Suistomaa *et al.*, 1987). Reduced glutathione (GSH), glutathione reductase and NADPH were obtained from Boehringer Mannheim (Mannheim, West Germany), while *t*-butyl hydroperoxide, cumene hydroperoxide, *o*-phenanthroline, iodoacetamide and glutathione (reduced) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Other reagents of analytical grade purity were products of E. Merck AG (Darmstadt, West Germany). Sephacryl S-300, molecular weight standards and Polybuffer 96 and 74 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

**Preparation of samples.** Human semen samples were obtained from donors admitted to the Fertility Clinic of Kuopio University Central Hospital. Only samples with normal sperm count ( $>20 \times 10^6/\text{ml}$ ) and motility ( $>40\%$  motility) were included. Spermatozoa were separated from seminal plasma by centrifugation at 600 *g* for 10 min. The seminal plasma was further centrifuged at 30 000 *g* for 1 h and the supernatant was used for biochemical fractionations. Human tissues were collected at autopsy from 5 adult human males within 2 days after death.

Semen was obtained from Ayrshire bulls by means of an artificial vagina at the AI Station for Eastern and Middle Finland and immediately sent to the laboratory in Dewar flasks. The semen samples were centrifuged at 30 000 *g* for 30 min at 4°C and the supernatants were taken for analysis. The reproductive tissues of bulls were obtained from Lihakunta Abattoir immediately after slaughter. After cleaning free of fat and connective tissue, the samples were homogenized in 0.025 M-imidazole-HCl buffer, pH 7.4, and centrifuged at 30 000 *g* for 1 h at 4°C. The tissue homogenates and isolated seminal vesicle secretion were used for selenium and GSH-Px assays as well as for biochemical fractionations.

**Fractionations.** Human and bovine seminal plasma samples and tissue homogenates were fractionated by gel filtration on Sephacryl S-300 columns (1.6 × 91 cm), anion exchange chromatography (Q Sepharose Fast Flow in HR 10/10 column) and chromatofocussing (Mono P column) as described by Agrawal & Vanha-Perttula (1986). Hydroxyapatite chromatography was carried out with Bio-Gel HPHT column (Bio-Rad, Richmond, CA, U.S.A.) eluted with a linear gradient of 0–0.2 M-phosphate buffer, pH 7.0. After elutions the fractions with high GSH-Px and/or selenium levels were pooled, dialysed and concentrated with Amicon ultrafiltration cell and PM-30 membrane (Amicon Corp., Lexington, MA, U.S.A.). The samples were then eluted in another fractionation system. The tentative molecular weights were estimated with gel filtration on Sephacryl S-300 or on Superose 6 column attached to a high-performance liquid chromatography system (h.p.l.c.: Altex, Berkeley, CA, U.S.A.) with molecular weight standards.

**Assay methods.** The selenium determination was performed using the graphite furnace technique, Zeeman background correction and pyrolytically coated graphite tubes with a platform (Zeeman 5000 Atomic Absorption Spectrophotometer, HGA 500; Perkin Elmer, Norwalk, CT, U.S.A.). The human and bull seminal plasma and tissues were treated as described previously (Saaranen *et al.*, 1986). The instrument conditions are presented in Table 1. The seminal plasma samples of men and bulls were diluted at 1:10 and 1:20, respectively, while the biochemical fractions were analysed undiluted. To each fraction 10  $\mu\text{l}$  0.19 M-Triton X-100 solution were added to help pipetting. A 20- $\mu\text{l}$  sample and 10  $\mu\text{l}$  of a 0.5% nickel  $[\text{Ni}(\text{NO}_3)_2]$  solution as a matrix modifier were pipetted into the furnace. The measurements were performed at 196.2 nm with a spectral band of 2.0 nm. The selenium concentrations were quantified using a matrix-based calibration curve. Peak area measurements were used in all analyses. Selenium content is given as ng/mg protein or ng/ml in fractionations.

GSH-Px was measured using the method of Günzler *et al.* (1974) with minor modifications. During preincubation (25°C for 3 min) the standard assay medium contained 500  $\mu\text{l}$  0.1 M-phosphate buffer (pH 7.0) with 1 mM-EDTA, 50  $\mu\text{l}$  1.125 M-Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 100  $\mu\text{l}$  glutathione reductase (2.4 U/ml), 100  $\mu\text{l}$  10 mM-reduced glutathione and 50  $\mu\text{l}$  of enzyme. After this 100  $\mu\text{l}$  1.5 mM-NADPH solution was added and the incubation was continued for 5 min at 37°C. The actual incubation was started by adding 100  $\mu\text{l}$  1.5 mM-hydrogen peroxide or 12 mM-*t*-butyl hydroperoxide. The decline of absorption at 340 nm was followed in a thermostated cuvette (37°C) of Stasar III spectrophotometer (Gilford Instrument Labs, Oberlin, OH, U.S.A.). The unit of enzyme activity is given as  $\mu\text{mol}$  NADPH oxidized/min per mg protein or per ml in tissue homogenates or fluids. The protein concentration was measured according to Bradford (1976) with bovine serum albumin as standard.

The optimum pH was measured in 0.1 M-phosphate (pH 5.0–8.0) and 0.1 M-Tris-HCl (pH 7.0–9.0) buffer series. In the modifier studies EDTA was omitted and replaced by various chemicals in 0.1 M-phosphate buffer. The thermal stability was tested by incubating the enzyme sample at various temperatures for 15 min or at 75°C for various periods

**Table 1.** The instrument conditions for selenium determination in human seminal plasma and in bull seminal plasma, tissue samples and biochemical fractions

Step	Human			Bull		
	Temp (°C)	Ramp (sec)	Hold (sec)	Temp (°C)	Ramp (sec)	Hold (sec)
Dry	90	10	15	100	10	15
	110	15	30	110	15	20
Charring	300	5	35	300	5	20
	1300	1	30	1100	1	30
	1300	1	4	1100	1	4
Atomization	2200	0	6	2300	0	6
	2650	1	3	2650	1	3
Cool	20	15	10	20	15	10

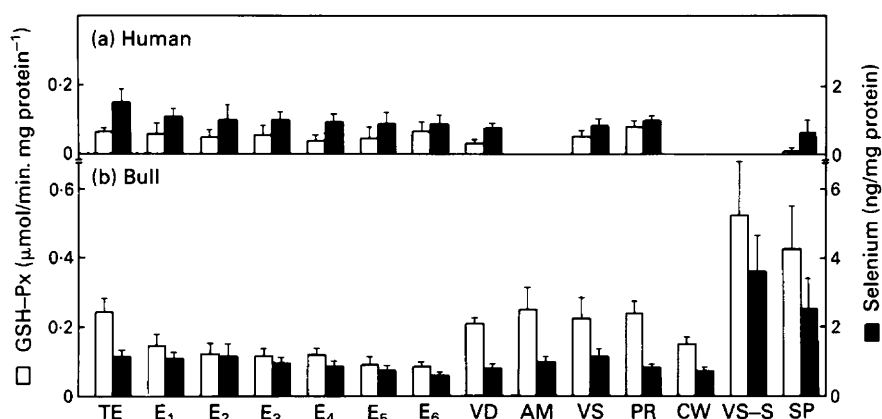
(0–60 min) before the actual incubation at 37°C. The results are given as percentage changes from the control without any modifier or without any heat treatment.

*Statistical analysis.* Student's *t* test was used to compare the mean values of selenium and GSH-Px in human and bovine samples. The analysis of variance was employed in comparing the various tissue concentrations in each species.

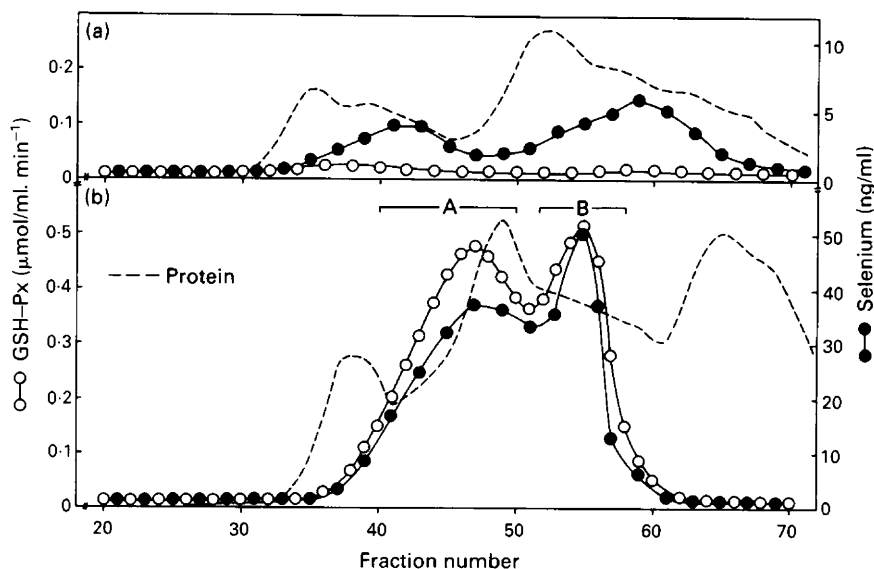
## Results

### Distribution

The distribution of selenium and GSH-Px with *t*-butylperoxide as substrate in the reproductive tissues of 5 human cadavers and in 5 bulls is shown in Fig. 1. In both species the tissue levels of



**Fig. 1.** The distribution of GSH-Px and selenium in homogenates of (a) human and (b) bull reproductive tissues. Each bar represents the mean  $\pm$  s.d. of 5 separate analyses. TE, testis; E<sub>1</sub>–E<sub>6</sub>, epididymal segments (E<sub>1</sub>–E<sub>3</sub>, caput; E<sub>4</sub>, corpus; E<sub>5</sub> and E<sub>6</sub>, cauda); VD, vas deferens; AM, ampulla; VS, vesicula seminalis; PR, prostate; CW, Cowper's gland; VS-S, seminal vesicle secretion; SP, seminal plasma.



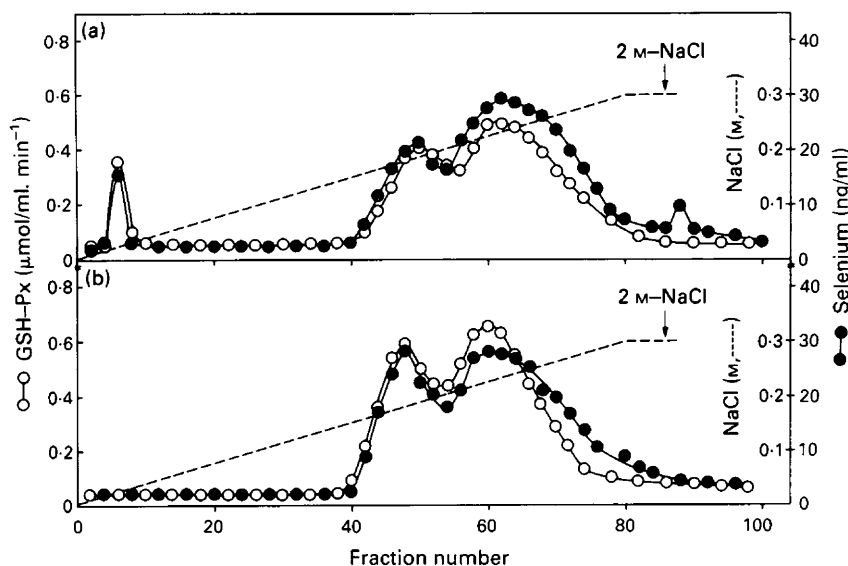
**Fig. 2.** Gel filtration of (a) human seminal plasma (5 ml) and (b) bull seminal plasma (5 ml) in Sephacryl S-300 column eluted with 0.02 M-Tris-HCl, pH 7.0 + 0.15 M-NaCl. Fractions of 2.2 ml were collected and GSH-Px and selenium were assayed of aliquants from the fractions as indicated in 'Materials and Methods'. The distribution of protein was measured from 10  $\mu$ l samples according to Bradford (1976) and the absorbance is given in the same scale with GSH-Px.

selenium were similar. The seminal plasma selenium concentrations were comparable to tissue levels in man, while in the bull the selenium concentrations were significantly ( $P < 0.001$ ) higher in seminal plasma and seminal vesicle secretion. GSH-Px displayed low and rather uniform levels in all human tissues, while in the bull various segments of the epididymis showed significantly ( $P < 0.05$ ) lower levels than the other tissues. Very low activities of GSH-Px were encountered in the human seminal plasma, while the bull seminal plasma and seminal vesicle secretion contained very high specific activities. With hydrogen peroxide as substrate an identical enzyme distribution was obtained (data not shown).

### Fractionation

In gel filtration on Sephacryl S-300, selenium was distributed in a wide area after fractionation of human seminal plasma (Fig. 2a). Two peak areas (Fractions 35–45 and 55–65) were usually seen. Hardly any GSH-Px activity could be detected in these fractions. On the other hand, gel filtration of bull seminal plasma resulted in two strong selenium and GSH-Px peaks eluting in Fractions 38–48 and 52–58, respectively (Fig. 2b). These fractions were separately pooled (A and B), concentrated and desalted with the Diaflo concentrator.

Anion exchange chromatography of Pools A and B with a linear NaCl (0–0.3 M) gradient from Q Sepharose FF column resulted in the appearance of two coincident areas for selenium as well as for GSH-Px (Fig. 3). With both enzyme samples the first peak ( $A_1$  or  $B_1$ ) eluted in Fractions 40–52 at 0.18 M-NaCl concentration, while the second peak ( $A_2$  or  $B_2$ ) was found in Fractions 56–75 at 0.22 M-NaCl. The relative sizes of these two peaks were about equal with those of samples A and B. The four active areas ( $A_1$ ,  $A_2$ ,  $B_1$ ,  $B_2$ ) were pooled, concentrated and desalted with Diaflo concentrator and dissolved in 2 mM-phosphate buffer, pH 7.0.



**Fig. 3.** Anion exchange chromatography on Q Sepharose FF column of (a) Pool A and (b) Pool B after gel filtration (see Fig. 2). The elution was carried out with a linear gradient (0–0.3 M) of NaCl. At the end of the gradient 2 M-NaCl was added to deplete the column. GSH-Px and selenium were measured in aliquants from the fractions (1 ml).

The four pools were separately applied for hydroxyapatite chromatography and eluted with a programmed gradient of phosphate buffer. Each sample resulted in two GSH-Px peaks at 0.14 M- and 0.16 M-phosphate concentrations (data not shown). Due to high phosphate concentration, the selenium analysis from the hydroxyapatite fractions could not be carried out. When the four pools were applied on a Mono P chromatofocussing column equilibrated with 0.025 M-ethanolamine-HCl buffer, pH 9.0, the GSH-Px activity and selenium eluted immediately at the beginning of the pH gradient (pH 9.0–6.0) developed by a combination of Polybuffer 96 and 74.

Gel filtration and anion exchange chromatography of bovine seminal vesicle secretion resulted in identical elution patterns as those obtained with seminal plasma. Tissue homogenates of ampulla, prostate and Cowper's glands gave a single peak of selenium and GSH-Px co-eluting with pool A in gel filtration.

### Enzyme characteristics

After gel filtration of bull seminal plasma, the optima for Pools A and B were both at pH 7.0 (Fig. 4). A similar result was obtained after further fractionation of these pools by anion exchange chromatography (Pools A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>, B<sub>2</sub>).

The effect of various modifiers on the pooled A and B activities are shown in Fig. 5. Both enzyme forms were extremely sensitive to heavy metals (Cd, Cu, Hg, Pb) and also to a variety of other divalent metal ions (Co, Fe, Mg, Mn, Ni, Zn) at 1 mM concentration. Cystein, dithioerythritol and iodoacetamide were also suppressive, while ethylenediamine tetra-acetate caused an activation. The other chelating agent, *o*-phenanthroline, however, suppressed both enzyme forms. At different concentrations the most effective inhibitor was Cu followed by Zn, Cd, Hg, sodium iodoacetate and iodoacetamide (Fig. 6a). No major differences were found between the two GSH-Px forms. Of the reducing agents cystein and dithioerythritol were about equally effective inhibitors, while glutathione caused a stimulation at various concentrations (Fig. 6b). In studies with Cu, Ni and Pb

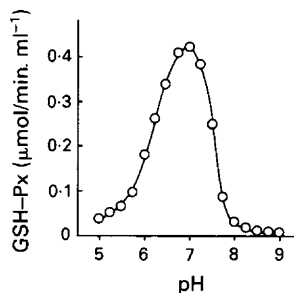


Fig. 4. The pH optimum for pooled Enzyme A after gel filtration (see Fig. 2).

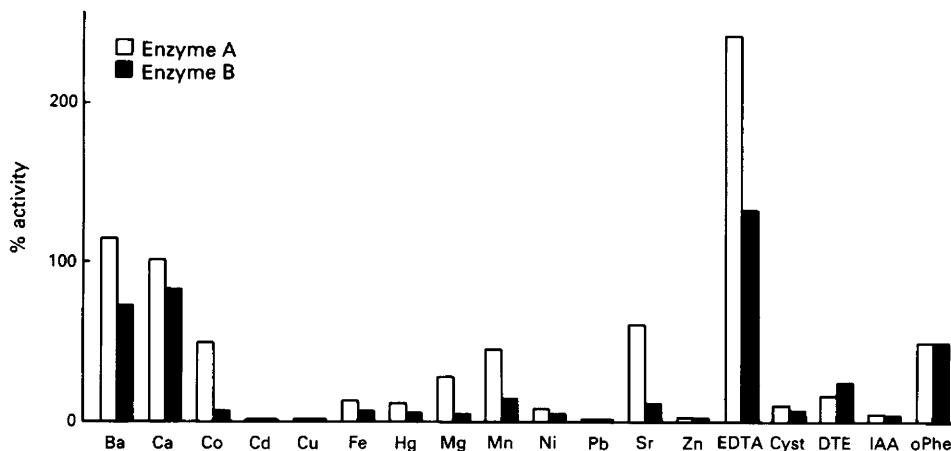
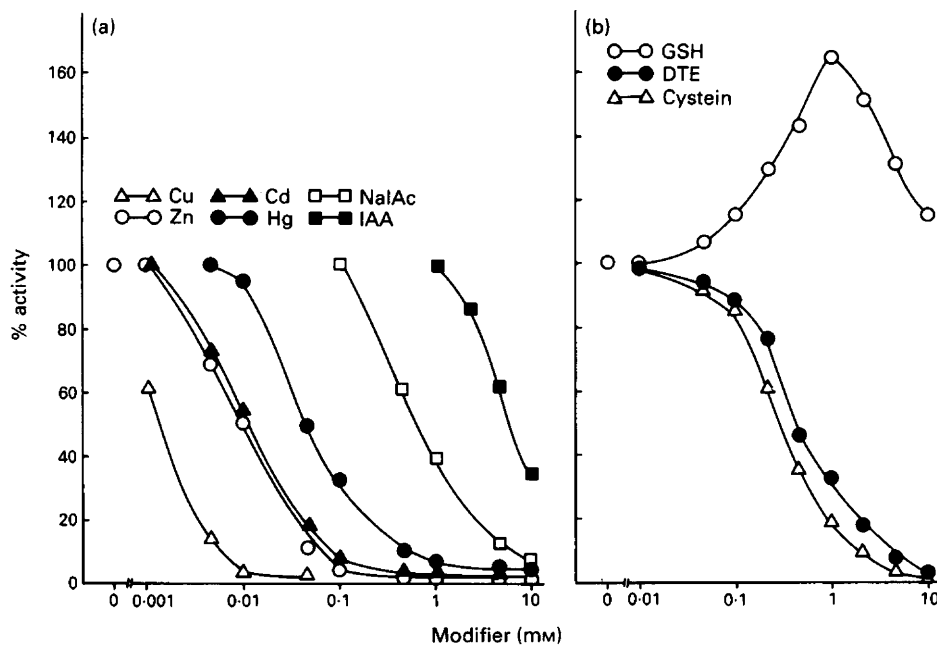


Fig. 5. The response of pooled Enzymes A and B after gel filtration (see Fig. 2) to various modifiers at the final concentration of 1 mmol/l. The results are given as % activity from the controls (100%) without any modifier. EDTA, ethylenediamine tetra-acetate; Cyst, cystein; DTE, dithioerythritol; IAA, iodoacetamide; oPhe, *o*-phenanthroline.

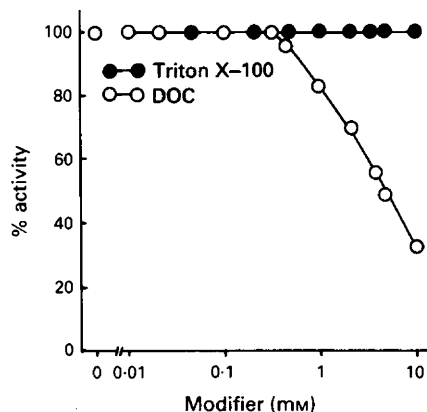
unusually high backgrounds were recorded at concentrations above 0.1 mmol/l. These were subtracted from the absorbances obtained with the enzyme samples present. Triton X-100 and sodium deoxycholate were tested at a concentration range of 0.01–10 mM. Triton X-100 had no effect on the enzyme activities, but deoxycholate at concentrations above 0.5 mM caused a clear suppression (Fig. 7).

When Pools A and B were exposed to various temperatures up to 70°C for 15 min hardly any suppression of the activities ensued. In a subsequent experiment Pools A and B were incubated at 75°C for various times and the remaining activities were measured. Pool A was clearly more resistant to the heat treatment than was Pool B activity (Fig. 8). Also, the A<sub>2</sub> and B<sub>2</sub> forms of GSH-Px were more resistant than A<sub>1</sub> and B<sub>1</sub> activities. In the presence of GSH (1 mM) the thermal inhibition could be effectively prevented even at 75°C. The protection was more complete for the B form of the enzyme.

When the activity of Enzymes A and B after gel filtration were assayed at different concentrations of *t*-butylperoxide, cumene hydroperoxide and hydrogen peroxide, the half-maximum activity ( $K_m$ ) was obtained at 0.8 mM, 0.4 mM and 0.2 mM levels, respectively. The corresponding maximum activities with both enzyme preparations were reached at 6, 4 and 0.4 mM concentrations of the substrates.

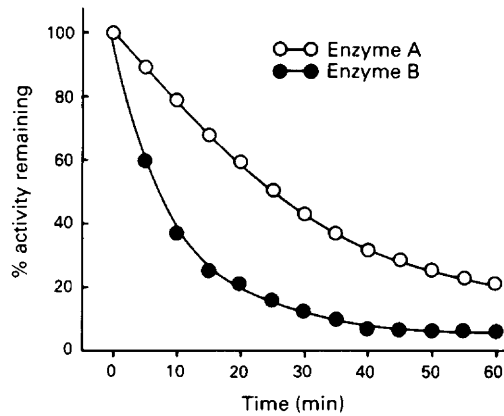


**Fig. 6.** Effect of (a) Cu, Zn, Cd, Hg, sodium iodoacetate (NaIAC) and iodoacetamide (IAA) and (b) of glutathione (GSH), dithioerythritol (DTE) and cystein at different concentrations on pooled Enzyme A (see Fig. 2). The results are given as % activity from the control (100%) without any modifier.



**Fig. 7.** Effect of Triton X-100 and sodium deoxycholate (DOC) at different concentrations on pooled Enzyme A (see Fig. 2). The results are given as % activity from the control (100%) without any modifier.

The tentative molecular weights of Pools A and B were estimated by gel filtration on Sephacryl S-300 column with standard proteins. The plotting of  $K_{av}$  against molecular weight resulted in values of 360 000 and 90 000 for the A and B forms, respectively. The molecular weight estimates obtained with Superose 6 column were comparable with these values.



**Fig. 8.** Thermal response of pooled Enzymes A and B after gel filtration (see Fig. 2). The enzyme samples were incubated at 75°C for various times, cooled and assayed for GSH-Px. The % activity in comparison to the controls (100%) without heat exposure was calculated.

### Discussion

Selenium has been found as a structural component of the mammalian GSH-Px molecule and therefore its measurement in tissues and body fluids has been considered to reflect the enzyme activity. Our previous study (Saaranen *et al.*, 1986) showed much lower selenium levels in human seminal plasma when compared to that of the bull. This would suggest that GSH-Px activity in human seminal plasma is also low. The present study, indeed, revealed substantial GSH-Px concentrations in bull seminal plasma and negligible values in human seminal plasma. This result was confirmed also after various fractionations, in which the elution patterns of selenium and GSH-Px were determined in the same samples. Both analyses were readily and reliably accomplished in all fractionations except for hydroxyapatite chromatography, in which the phosphate buffer elution interfered with selenium quantitation by atomic absorption spectrometry.

The tissue homogenization was carried out in hypotonic solution and the insoluble particulate material was removed by centrifugation. The strongly bound selenium in the sperm structures possibly remained in the pellet. This structural selenium can increase the amount of this element in testicular and epididymal tissue, if the assay is carried out by ashing or acid/base digestion of the crude tissue samples. This may be the reason for the relatively higher selenium levels in human and bovine testis and epididymis in our previous study (Saaranen *et al.*, 1986) in comparison to the present findings, which gave rather uniform selenium concentrations in all reproductive tissue homogenates.

In man GSH-Px was not secreted into the seminal plasma and the tissue levels were also lower than those in the bull. The tissue samples taken from cadavers also contained some blood, which may have affected the GSH-Px measurements. Blood contamination may also have influenced the selenium values obtained for human reproductive organs. The selenium content of the seminal fluid indicates that this element is somehow released from the reproductive organs. It was bound to macromolecules, which did not give any GSH-Px activity in fractionations. The macromolecules may originate from any part of the reproductive system. They could even be structural molecules of spermatozoa disintegrated during epididymal transit.

High selenium and GSH-Px concentrations were characteristic for bovine seminal plasma. Comparable values were also recorded in the seminal vesicle secretion, which indicates a major role of this gland in their secretion. We cannot exclude, however, that other parts of the reproductive system (e.g. vas deferens, ampulla, prostate and Cowper's glands) are contributing as well. This



possibility was substantiated by fractionation studies, which revealed co-eluting selenium and GSH-Px in homogenates of seminal vesicles as well as other accessory sex glands.

GSH-Px and selenium co-eluted in all fractionations of bull seminal plasma. In gel filtration two macromolecules were disclosed, which differed from each other in molecular weights. The larger GSH-Px had a molecular weight about four times that of the smaller one. It is possible that the latter forms the basic enzyme unit, which is readily assembled into a tetramer. Such an assembly/disassembly could be visualized in anion exchange chromatography of the two molecular forms of GSH-Px. The tetramer form appeared to correspond to the enzyme, which was more firmly attached to the anion exchange column. Partial separation of two enzyme forms was also obtained in hydroxyapatite chromatography, while neither of them was attached to the chromatofocussing column.

The two enzyme forms had equal pH optima and inhibition by some heavy metals (Cd, Cu, Hg, Pb), divalent metal ions (Co, Fe, Mg, Mn, Ni, Zn) and reducing agents (cystein, dithioerythritol). Both enzyme forms were activated by EDTA and GSH. However, these GSH-Px activities differed from each other in thermal stability. The tetramer form was more resistant to heat treatment. The reason for this difference could be due to distinct exposure of reactive groups in the two forms of the enzyme. It may also depend directly on the stability of selenium in the active centre of the mono- and tetrameric enzymes. Human erythrocytes have been found to contain two forms of GSH-Px, which differ from each other in hydrophobic surface properties (Forward & Almog, 1985). In the present study the two forms of the enzyme were not influenced by Triton X-100, while deoxycholate was clearly suppressive. A response similar to that of these two substances has recently been described for a phospholipid hydroperoxide GSH-Px (Ursini *et al.*, 1985; Maiorino *et al.*, 1986). It may, indeed, be possible that the bovine seminal plasma enzyme is identical with the latter and not with the classical GSH-Px isolated from erythrocytes.

GSH-Px may provide a mechanism which protects the sperm membrane from oxidative damage and subsequent cell death (Senger, 1980). In the bull, with high GSH-Px values in the seminal plasma, such a function is acceptable. This enzyme may also contribute to the good viability of bovine spermatozoa over prolonged time periods at room temperature and in various steps of cryopreservation. Human spermatozoa are more susceptible to such conditions but still survive freezing in appropriate extenders. Mechanisms other than GSH-Px must provide the protection for the human sperm plasma membrane against oxidative damage. The establishment of roles of GSH-Px and selenium in the viability of spermatozoa would require further comparative studies as well as other experimental approaches.

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