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A SELENOENZYME, GLUTATHIONE PEROXIDASE FROM HUMAN PLACENTA: PURIFICATION AND SOME PROPERTIES*

Glutathione peroxidase (GSH-Px) from human placenta was purified approximately 2200 times. Ammonium sulfate (25–60% saturation) precipitation, Sephadex gel filtration and DEAE-Sephadex A–50 chromatography were used for purification procedures. The enzyme was shown to be homogenous by polyacrylamide-gel electrophoresis. The molecular weight (78 000 daltons), isoelectric point (5.50), optimum pH (7.8–8.0) and temperature (42°C), were determined. Iodoacetate, N-ethyl maleimide and cyanide inhibit the enzyme activity by 80–90%. Dithiothreitol, GSH and 2-mercapto-ethanol protect the enzyme from inactivation. Divalent cations inhibit the activity of GSH-Px.

1. INTRODUCTION

Glutathione peroxidase (GSH-Px; glutathione: hydrogen peroxide oxidoreductase; EC 1.11.1.9) discovered in 1957 by Mills [15] is up to now the only known mammalian selenium-dependent enzyme [5, 20]. GSH-Px catalyses the reduction of H_2O_2 and organic hydroperoxides to water and corresponding alcohols with the reduction of GSH. It is a primary cellular antioxidative enzyme and serves to prevent the formation of toxic oxygen species. Attempts were made to purify this enzyme from human [1], bovine [24] and other animal red cells [13, 19, 20] or tissues [2, 16]. GSH-Px consists of 4 identical subunits of 21 000 daltons and contains 4 atoms of selenium per native enzyme molecule [5]. Selenium is present at the active sites as selenocysteine residue [6] localized at positions 41–47 from N-terminal end of the protein [22]. The refined three-dimensional structure of the enzyme has been studied by Epp et al. [4] and the amino acid sequence as well as the mechanism of selenium incorporation has been elucidated [9, 17]. It has been shown [10] that human

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placenta does not constitute a barrier to the movement of seleno-amino acids and accumulated seleno-methionine, the natural organic form of selenium. Inorganic selenium and seleno-methionine are utilized in various mammalian tissues for GSH-Px synthesis [23].

The aim of the present study was to isolate the GSH-Px from human placenta and to compare some of its properties with those of the enzyme isolated from other mammalian sources.

2. MATERIALS AND METHODS

2.1. Chemicals

Sephadex and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, GSH reductase, iodoacetate and EDTA were purchased from Sigma Chem. Co., USA. GSH, acrylamide, t-butyl- and cumene hydroperoxide were kindly supplied by Koch-Light Labs, England. All other chemical used were of analytical grade purity.

2.2. Purification procedures

All purification procedures were conducted at 0–4°C. Fresh human placentae were used after a physiological delivery from healthy women. The tissue was cut into pieces, washed in cold buffer containing 0.9% NaCl and 5 mM Tris-HCl, pH 7.6 (1: 1; vol: vol) and homogenized. The homogenate was centrifuged at 3000 g for 10 min, the supernatant was transferred and centrifuged at 100 000 g for 1 h. To the clear supernatant solid $(NH_4)_2SO_4$ was added. The precipitate obtained in the range of 25–60% saturation was used for further purification steps. Precipitated protein was dissolved in a small volume of 10 mM Tris-HCl buffer, pH 7.6 and dialysed against the same buffer for 24 h. The enzyme was centrifuged and the supernatant was applied into a Sephadex G-100 column (2.5 × 100 cm) and eluted with a Tris-HCl buffer, pH 7.6. Additionally, the following purification procedures were used [1, 25, 26] with slight modifications:

a) DEAE-Sephadex A-50 column (2.5 × 30 cm) and 0.05–0.50 M NaCl in 10 mM Tris-HCl buffer, pH 6.5 as eluent.

b) G-150 column (2.5 × 100 cm) and 10 mM Tris-HCl buffer, pH 7.6 as eluent.

c) DEAE-Sephadex A-50 column (1 × 20 cm) and the same buffer as in a)

d) additional chromatography and the same ion-exchanger and buffer were used. Points a), c) and d) are numbered as DEAE-Sephadex A–50 I, II and III.

2.3. Polyacrylamide gel electrophoresis

Disc gel electrophoresis was performed by the method of Laemli [12]. The isoelectric point was determined according to the method of Gronow and Griffith [8] using 4% polyacrylamide gel with 2.4% of Ampholine, pH 3–6. The protein was stained with Coomassie Brilliant Blue.

2.4. GSH-Px activity and protein concentration determinations

GSH-Px activity was assayed by the method of Paglia and Valentine [18] with t-butyl hydroperoxide as substrate. Protein concentration in the eluates was determined spectrophotometrically, and additionally in the GSH-Px peaks by the method of Lowry et al. [14].

3. RESULTS

3.1. Purification of GSH-Px

The results of a typical purification procedure are summarized in Table 1. The initial GSH-Px activity of the ammonium sulfate fraction varied between 0.013–0.060 enzyme units. The results achieved in particular experiments

Table 1

Summary of purification procedure of glutathione peroxidase

Step	Protein concn (mg)	Total activity* (units)	Specific activity	Purification (fold)	Yield (%)
Supernatant	7340	235	0.032	1.0	100
$(NH_4)_2SO_4$ (25–60%)	929	84.5	0.091	2.8	69.3
Sephadex G-100	62	54.3	0.874	27.5	23.1
DEAE-Sephadex A-50 I	15.1	46.5	3.08	97.1	19.8
Sephadex G-150	1.6	40.3	25.2	793	17.2
DEAE-Sephadex A-50 II	0.6	30.1	50.3	1587	12.8
DEAE-Sephadex A-50 III	0.1	7.0	70.4	2221	8.9

* One unit of GSH-Px is the amount of NADPH oxidized/min/mg protein (25°C).

varied very considerably. The degree of final purification of the enzyme varied from 1660 to 2550 times with the specific activity between 22.3–118.3 U/mg protein and the yield equalled 3–17%. A typical chromatographic elution pattern of GSH-Px from Sephadex G-150 and DEAE-Sephadex A-50 III is presented in Fig. 1. As can be seen from this figure (upper part), in a wide absorbance at 280 nm there is a sharp peak of GSH-Px. The lower part of the figure shows that the GSH-Px fraction is almost homogenous. This fraction of GSH-Px was used in polyacrylamide gel electrophoresis for checking the homogeneity and the molecular weight (Fig. 2). The GSH-Px preparation moved in polyacrylamide disc gel electrophoresis as a one protein band.

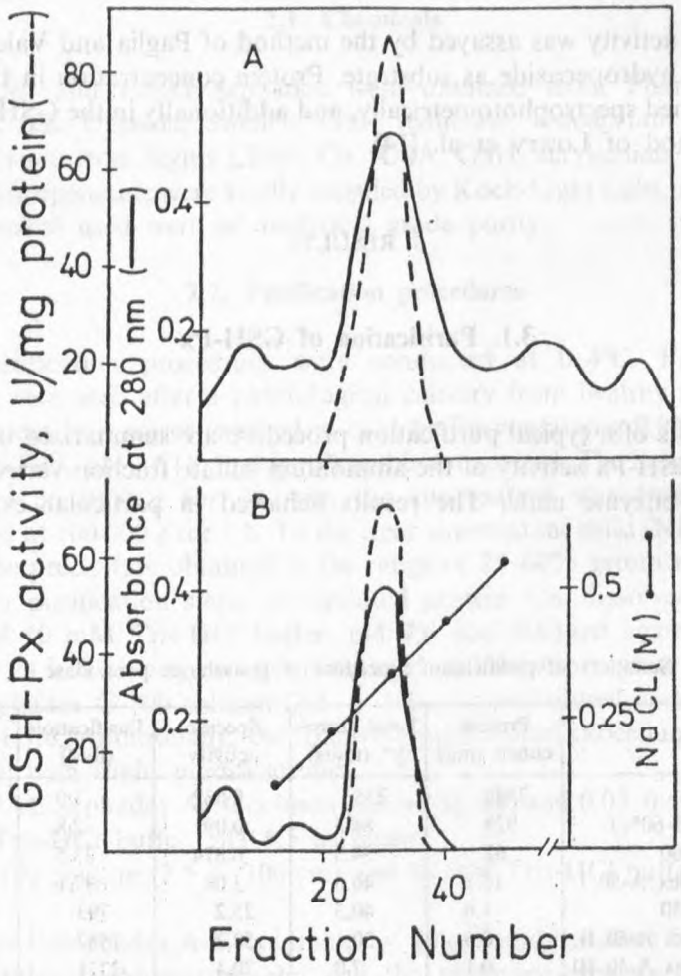

Fig. 1. Sephadex G-150 gel filtration (upper part) and DEAE-Sephadex A-50 (III) chromatography (lower part) of human placental GSH-Px

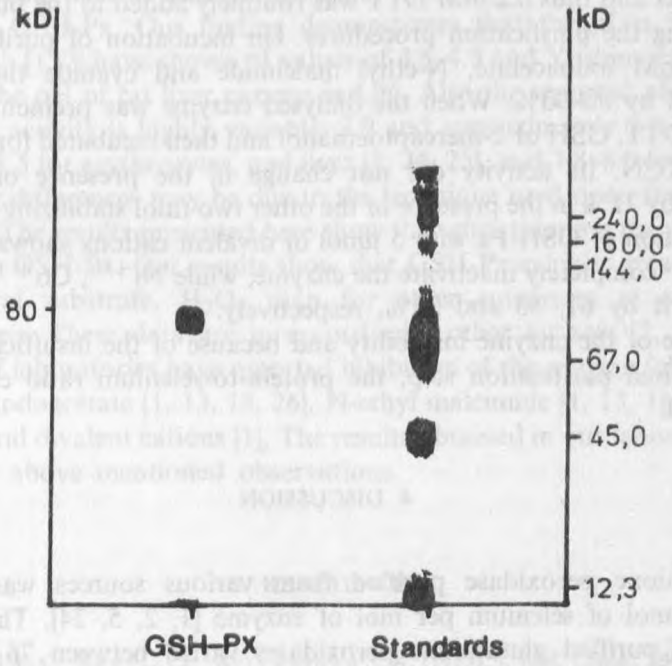

Fig. 2. Polyacrylamide disc gel electrophoresis of purified GSH-Px from human placenta (left) and standard proteins (right). From top to bottom: bovine liver catalase (240 000), rabbit muscle aldolase (160 000), rabbit muscle dehydrogenase (144 000), human serum albumin (67 000), ovoalbumin (45 000) and cytochrom c (12 500). The molecular weight of the isolated placental GSH-Px is 78 000

3.2. Properties of placental glutathione peroxidase

The molecular weight of the purified enzyme, as determined by polyacrylamide gel disc electrophoresis, was 78 000 daltons. The isolated enzyme focused as a single peak at pH 5.50. The activity of the GSH-Px was measured at a pH range of 6.2–8.6 and at temperatures between 25–50°C. The optimum pH was established to be 7.8–8.0, and the optimum temperature, 42°C. The enzyme appeared to be specific for reduced glutathione, as replacement by L-cysteine and cysteamine showed only 9.1 and 0.9% activity, respectively. Among the hydroperoxides tested the activity observed was as follows: H_2O_2 > cumene hydroperoxide > t-butyl hydroperoxide (100, 85.2 and 69.6%, respectively). The K_m established for t-butyl hydroperoxide was 78.5 μM. We observed that the purified GSH-Px was much less stable as compared to the crude enzyme preparation. The addition of dithiothreitol (DTT) or GSH stabilizes the enzyme both at +4°C and at –30°C [7]. Dithiothreitol exerts

better effect and thus 0.2 mM DTT was routinely added to the buffer solution used during the purification procedures. On incubation of purified GSH-Px with 10 mM iodoacetate, N-ethyl maleimide and cyanide the enzyme is inactivated by 80–90%. When the dialysed enzyme was preincubated for 10 min with DTT, GSH or 2-mercaptoethanol and then incubated for 60 min with 33 mM KCN, its activity did not change in the presence of DTT, and decreased by 21% in the presence of the other two thiol stabilizing compounds.

Incubation of GSH-Px with 5 μmol of divalent cations shows that Hg^{++} and Cu^{++} completely inactivate the enzyme, while Ni^{++}, Co^{++} and Ca^{++} inactivate it by 61, 53 and 23%, respectively.

Because of the enzyme instability and because of the insufficient material after the final purification step, the protein-to-selenium ratio could not be determined.

4. DISCUSSION

Glutathione peroxidase purified from various sources was shown to contain 4 mol of selenium per mol of enzyme [1, 2, 5, 24]. The molecular weights of purified glutathione peroxidases varied between 76 000–97 000 daltons [1, 2, 11, 21, 24]. To the best of our knowledge only one report has been published on the GSH-Px molecular weight, purified from bovine lens, to be as heigh as 140 000 daltons [3]. The purification methods used in our experiments yielded a 2200-fold purification of the enzyme. The degree of purification described by other authors was in most cases almost in the same range [13, 24, 25]. Different molecular weights, and various purification folds of the homogenous fraction were probably due to different analytical techniques used in their experiments. The enzyme purified by us had a specific activity of 70.4 U/mg protein, which is higher than that purified from bovine lens [11] but significantly lower than the enzyme purified from human erythrocytes with specific activity of 103 U/mg protein [1] or that purified from other sources with much higher specific activities [2, 3, 13, 21, 24–26]. The reported stability of the purified GSH-Px was highly variable. Our result is in accord with Little et al. [13] who observed that the less purified samples of the enzyme were much more stable to storage than purified GSH-Px. Awasthi et al. [1] have shown that the erythrocyte enzyme is stable at pH 7.0 at 4°C but very unstable at low pH values. At pH 4.0 the enzyme inversely lost all the activity in 20 min. Bergad et al. [3] demonstrated that concentrated (more than 100 units/ml) lens enzyme preparations lost no activity for 6 months when stored at –20 to –80°C. However, diluted samples (less than 1 units/ml) lost about 65% of their activity when kept 3 hours at –20°C. In the presence of bovine serum albumin the enzyme activity was stabilized.

Reports from several laboratories differ in the values of isoelectric pH of the purified GSH-Px. Our finding demonstrates that the pI is 5.50, while Awasthi et al. [1, 2] have shown pI values of 4.8–4.9 and Yoshimura et al. [25] found that the pH of rat liver enzyme is 6.96. Also the reported pH optimum for GSH-Px activity is highly variable: 7.9 and approximately 9.0 for bovine lens [3, 11], 8.5 for erythrocytes, and liver [1, 24, 25], and 7.8–8.0 found in this study. These differences may be due to the technique used or to the source of the enzyme. The results presented here show that glutathione is the most active substrate for GSH-Px. Our results show that GSH-Px exhibits greater affinity for a natural substrate, H_2O_2 than for other substrates at comparable concentrations. These data are in accord with other authors [2, 3, 11, 26]. A number of laboratories have reported inhibition of the enzyme following the addition of iodoacetate [1, 13, 18, 26]. N-ethyl maleimide [1, 13, 18], KCN [2, 19] and several divalent cations [1]. The results obtained in our laboratory fully confirm the above mentioned observations.

5. REFERENCES

[1] Awasthi Y. C., Beutler E., Srivastava S. K. (1975), J. Biol. Chem., **250**, 5144–5149.
[2] Awasthi Y. C., Dao D. D., Lal A. K., Srivastava S. K. (1979), Biochem. J., **177**, 471–476.
[3] Bergad P. L., Rathbun W. B., Linder W. (1982), Exp. Eye Res., **34**, 131–144.
[4] Epp O., Ladenstein R., Wendel A. (1983), Eur. J. Biochem., **133**, 51–69.
[5] Flohe L., Gunzler W. A., Schock H. H. (1973), FEBS Lett., **32**, 132–134.
[6] Forstrom J. W., Zakowski J. J., Tappel A. L. (1978), „Biochemistry", **17**, 2639–2644.
[7] Gromadzińska J., Zachara B. (1980), Biul. WAM (Poland), **23**, 110–115.
[8] Gronow M., Griffith B. (1971), FEBS Lett., **15**, 340–344.
[9] Gunzler W. A., Steffens G. J., Grossmann A., Kim S.-M. A., Otting F., Wendel A., Flohe E. (1984), Hoppe-Seyler's Z. Physiol. Chem., **365**, 195–212.
[10] Hansson E., Jacobsson S. (1966), Biochim. Biophys. Acta, **115**, 285–293.
[11] Holmberg N. J. (1968), Exp. Eye Res., **7**, 570–580.
[12] Laemli U. (1970), „Nature", **227**, 680–685.
[13] Little C., Olinescu R., Reid K. G., O'Brien P. J. (1970), J. Biol. Chem., **245**, 3632–3636.
[14] Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J. (1951), J. Biol. Chem., **193**, 265–275.
[15] Mills G. C. (1957), J. Biol. Chem., **229**, 189–197.
[16] Miwa T., Adachi T., Ito Y., Hirano K., Sugiura M. (1983), Chem. Pharm. Bull., **31**, 179–183.
[17] Mizutani T., Hitaka T. (1988), FEBS Lett., **232**, 243–248.
[18] Paglia D. E., Valentine W. N. (1967), J. Lab. Clin. Med., **70**, 158–169.
[19] Prohaska J. R., Oh S. -H., Hoekstra W. G., Ganther H. E. (1977), Biochem. Biophys. Res. Commun., **74**, 64–71.

[20] Rotruck J. T., Pope A. L., Ganther H. E., Swanson A., Hoekstra W. G. (1973), „Science", **179**, 588–590.
[21] Stults F. H., Forstrom J. W., Chiu D. T. Y., Tappel A. L. (1977), Arch. Biochem. Biophys., **183**, 490–497.
[22] Sunde R. A., Evenson J. K. (1987), J. Biol. Chem., **262**, 933–937.
[23] Whanger P. D., Beutler J. A. (1988), J. Nutr., **118**, 846–852.
[24] Yagi K., Kondo N., Ohkawa H., Ohishi N. (1980), Biochem. Internat., **1**, 245–252.
[25] Yoshimura S., Komatsu N., Watanabe K. (1980), Biochim. Biophys. Acta, **621**, 130–137.
[26] Zakowski J. J., Tappel A. L. (1978), Biochim. Biophys. Acta, **526**, 65–76.

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PEROKSYDAZA GLUTATIONOWA — SELENOZALEŻNY ENZYM Z ŁOŻYSKA LUDZKIEGO: OCZYSZCZANIE I NIEKTÓRE WŁAŚCIWOŚCI

Peroksydazę glutationową (GSH-Px) z łożyska ludzkiego oczyszczono ok. 2200-krotnie, stosując następujące techniki: wytrącanie siarczanem amonowym (25-60% nasycenia), chromatografia na Sephadex G-100, Sephadex G-150 oraz DEAE-Sephadex A-50. Elektroforeza w żelu poliakrylamidowym wykazała istnienie jednorodnej frakcji GSH-Px. W oczyszczonym enzymie oznaczono masę cząsteczkową (78 000 daltonów), punkt izoelektryczny (5,50), optimum pH (7,8–8,0) i optimum temperatury (42°C). Jodooctan, N-etylenomaleimid i cyjanki inhibują aktywność enzymu w 80–90%. Ditiotreitol, glutation i 2-merkaptoetanol chronią enzym przed inaktywacją. Dwuwartościowe kationy są również inhibitorami GSH-Px.