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Purification and Characterization of Thiol-Specific Antioxidant Protein from Human Liver: A Mer5-Like Human Isoenzyme

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Abstract: A 23-kDa molecular mass of antioxidant protein was purified from human liver. This protein exhibited the preventive effect against the inactivation of glutamine synthetase by a metal-catalyzed oxidation system. This antioxidant activity was supported by a thiol-reducing equivalent such as dithiothreitol in a similar manner to that of the 25-kDa thiol-specific antioxidant protein (TSA) from human red blood cells (HR). However, a thioredoxin-linked peroxidase activity of thiol-specific antioxidant protein of human liver (HLTSA) (0.91 μmol/min/nmol of HLTSA) was much lower than that of thiol-specific antioxidant protein of human red blood cells (HRTSA) (16.4 μmol/min/nmol of HRTSA). This HLTSA is also immnologically distinct from HRTSA. Amino acid sequences of the three tryptic peptides (P1, P2, P3) of HLTSA were found to be completely homologous to segments of the known Mer5-like protein, which belongs to the known TSA family.

Key words: antioxidant, human, isoenzyme, liver, thiol-specific.

Reactive oxygen species (O^{-}_2, H_2O_2, ROOH, and HO•) are generated by many physiological process such as incomplete reduction of molecular oxygen during respiration, NADPH oxidation linked to respiratory burst during phagocytosis, and redox cycling of xenobiotics (Halliwell *et al.*, 1989). Excess reactive oxygen species can damage cells. To prevent the deleterious effect of oxygen species, cells have been equipped with a number of antioxidant enzymes, including catalases, peroxidase, and superoxide dismutases.

A new type of antioxidant enzyme family, called thiol-specific antioxidant protein (i.e., TSA or PRP) family, is fast growing (Chae *et al.*, 1994). These enzymes prevent the oxidative damage induced by the metal-catalyzed oxidation (MCO) system, whose activities are supported by a thiol reducing equivalent such as DTT (Kim *et al.*, 1988) or an *in vivo* thiol reducing equivalent such as thioredoxin (Trx) (Chae and Chung *et al.*, 1994; Kwon *et al.*, 1994). The enzymes and their genes were identified from all life kingdoms. Recently we purified a member of TSA from human RBC (Lim *et al.*, 1994) and identified the enzyme as a type of *human brain* TSA (Cha *et al.*, 1995). Analysis of homology search in data base (BLASTP 1.4.8 M) using the amino sequence of human brain TSA gene product (Lim, Cha and Kim *et al.*, 1994) revealed that there are three types of TSA isoenzymes in human tissues including human RBC TSA or brain TSA. The gene of a second human form called the *PAG* gene product was cloned and sequenced. Prosperi *et al.* (1993) reported the 22-kDa *PAG* gene product, the second human TSA isoenzyme. The expression of *PAG* gene increases during proliferation of human epithelial cells. The *Mer5* gene from murine erythroleukemia (Yamamoto *et al.*, 1989) is preferentially expressed in murine erythroleukemia cells and suggests a possible role in the induction of differentiation. The human Mer5-like gene, a possible third type of isoenzyme, was cloned and sequenced (Tsuji *et al.*, 1995). Watabe *et al.* (1994) reported a substrate protein, SP-22, for mitochondrial matrix ATP-dependent protease in bovine adrenal cortex. By a homology search in the data base, SP-22 was found to be homologous to murine erythroleukemia cell *Mer5* protein. However, there is no report on the purification and characterization of the Mer5-like protein in human tissues, a strong candidate for a third type of human TSA isoenzyme.

In this communication, we report, for the first time, the purification and characterization of thiol-specific antioxidant protein from human liver (HLTSA), and identification of this enzyme as a third type of human iso-

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enzyme, Mer5-like protein.

Materials and Methods

Materials

A stock solution of $FeCl_3$ was prepared in 0.1 N HCl. Glutamine synthetase was purified from the *E. coli* (Pgln/YMC10) as described (Rhee *et al.*, 1985). HRTSA (HRPRP) was purified from human RBC as the method previously described (Lim *et al.*, 1994). Two protein components, thioredoxin and thioredoxin reductase also were purified from wild type *E. coli* K12 (Tsang and Weatherbee, 1981).

Determination of thiol dependent antioxidant activity

Thiol-specific antioxidant activities of enzymes were determined by monitoring their activities to inhibit the inactivation of *E. coli* glutamine synthetase (GS) by a metal catalyzed thiol MCO system (DTT, Fe^{3+}, O_2) (Stadtman and Oliver, 1991) as described by Kim *et al.* (1988). Instead of DTT, ascorbate was included as a non-thiol reducing equivalent (non-thiol MCO system). Fifty μl of reaction mixture containing 5 μg of glutamine synthetase, 3 μM $FeCl_3$, 10 mM DTT or ascorbate, antioxidant enzyme, and 100 mM Hepes-NaOH (pH 7.0) were incubated at 37℃ for 60 min. The remaining activity of glutamine synthetase was measured by the addition of 10 μl of the reaction mixture to 2 ml of γ-glutamyltransferase assay mixture as described (Kim et al., 1988).

Purification of TSA from human liver

To remove blood contained in human liver, the liver was perfused with phosphate-buffered saline (PBS) buffer until the perfusate became colorless. The chopped liver tissues (500 g) were washed three times with the PBS buffer. The liver tissues were homogenated in 50 mM Tris-HCl buffer, pH 7.6, using Waring blender and centrifuged at 28,000×g for 60 min twice. After centrifugation, the clear supernatant was loaded into a DEAE column. The column was washed with equilibrium buffer (50 mM Tris-HCl buffer, pH 7.6) and eluted with a linear KCl gradient (0~400 mM). TSA activity was assayed throughout the purification by monitoring its ability to prevent GS inactivation by a thiol MCO system. Broad peak fractions of TSA activity eluted at a KCl concentration between 250 and 400 mM were pooled and precipitated with 70% ammonium sulfate. The dissolved ammonium sulfate-precipitate was applied to a Sephacryl S-200 gel permeation column (2.5×50 cm) previously equilibrated with 100 mM Hepes buffer, pH 7.4, containing 100 mM KCl.

Determination of peroxidase activity of the antioxidant enzyme linked to Trx

To determine peroxidase activities of HLTSA or HRTSA-linked to NADPH oxidation, the reaction was started by the addition of various amounts of the antioxidant enzyme to 50 mM Hepes-NaOH buffer, pH 7.0, containing 0.25 mM NADPH, varying concentrations of peroxides, 12.5 μg/ml Trx, and 12.5 μg/ml TR. The resulting oxidation of NADPH was directly followed by the decrease in absorbance at 340 nm.

Sequencing of tryptic peptides from HLTSA

Two mg of the purified 23-kDa HLTSA was reductively denaturated by a 6 M guanidine hydrochloride solution containing 1 mM DTT and 50 mM Tris-HCl (pH 7.8). The sulfhydryl group(s) was labeled with TNB by 10 mM DTNB for 1 h at 37℃ (Sliwkowski *et al.*, 1985). The TNB-linked protein was precipitated with 10% TCA, and the precipitate was washed three times with acetone. The resulting protein was suspended in 50 mM Tris-HCl (pH 7.6), and after digestion with 20 g of trypsin for 3 h at 37℃, additional digestion with another 20 μg of trypsin was carried out overnight at 30℃. The resulting peptides were applied to a preparative Vydac C_{18} column (25×250 mm), and eluted with a linear gradient of 0~60% acetonitrile in 0.05% trifluoroacetic acid for 60 min at a 2 ml/min of flow rate.

Other methods

Immunoblot analyses of HLTSA and HRTSA were performed by using rabbit polyclonal antibodies against yeast TSA. Procedures for transfer of proteins from SDS-polyacylamide gels to nitrocellulose and for the processing of nitrocellulose blots have been described previously (Kim *et al.*, 1989). Protein concentration was determined by using the Coomassie Brilliant blue G-250 Protein Assay kit (BioRad, Richmond, USA) based on the method of Bradford (1976). SDS-PAGE was performed by the method of Laemmli (1970).

Results and Discussion

Purification of TSA from human liver

We purified a thiol dependent antioxidant protein from the whole extract of human liver (HLTSA) to homogeneity by two subsequent chromatographic steps on DEAE and Sephacryl S-200 HR chromatography (Fig 1). To simplify the nomenclature, this antioxidant protein will be subsequently designated HLTSA. The DEAE cellulose chromatography yielded broad activity peaks between 250 and 400 mM of KCl gradients

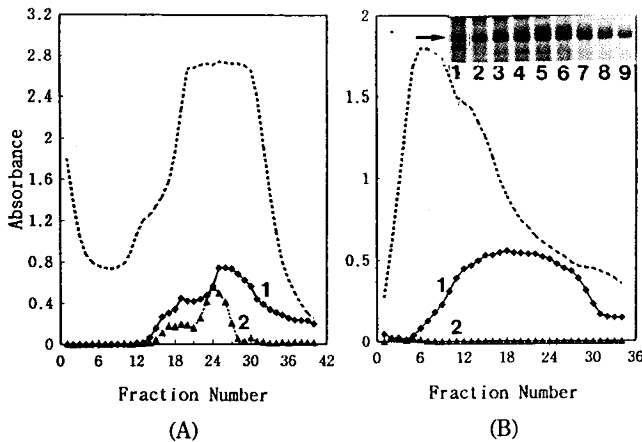

Fig. 1. Purification of the thiol-specific antioxidant protein from human liver (HLTSA). Detailed procedures on column chromatography steps are described under Materials and Methods. (A), DEAE-cellulose chromatography;(B), gel filteration chromatography on Sephacryl S-200. Dotted line, protein peaks measured at 280 nm; curves 1 and 2 in the figure, the remaining glutamine synthetase activity sujected to thiol MCO and non-thiol MCO systems, respectively. Lanes from 1 to 9 inset of Fig. 1-B; 12% reducing SDS-PAGE of the fractions 12, 14, 16, 18, 20, 22, 24, 26 and 28, respectively. 40 μl of samples were precipitate with 10% TCA, and applied to the SDS-PAGE. An arrow in the inset indicates 23-kDa HLTSA.

(Fig. 1-A). The active fractions between fraction 28 and 40 were collected and loaded onto a Sephacryl S-200 gel permeation chromatographic column. The antioxidant activity appeared from the shoulder just after the major protein peak, showing a rather wide activity peak (Fig. 1-B). Each fractions of the S-200 column containing TSA activity were subjected to 12% SDS-PAGE. Fig. 1-B and its inset show that both the TSA activity and the intensity of the 23-kDa protein band peaked around fraction 20. The fractions containing the TSA (fractions between 22 and 28) were collected and subjected to 12% SDS-PAGE. Lane 2 of Fig. 2-A shows the purity of HLTSA. The purified protein was analyzed on SDS-PAGE and visualized by both Coomassie blue staining (Fig. 2-A) and western blot using polyclonal antibodies against yeast TSA (Fig. 2-B). The reducing and nonreducing SDS-PAGE analysis for HLTSA (lane 2 and 4, respectively) showed that HLTSA exists in dimers linked to each other *via* an intermolecular disulfide linkage as in the case of HRTSA (lane 3 and 5) (Lim *et al.*, 1994). The different cross-reactivities of HLTSA and HRTSA to yeast TSA antibody (Fig. 2-B) indicate that HLTSA is a new type of isoenzyme of Human TSA. The molecular weights of HLTSA were determined to be 23-kDa on reducing SDS-PAGE (12 %) with β-mercaptoethanol.

Amino acid sequence of tryptic peptides of HLTSA

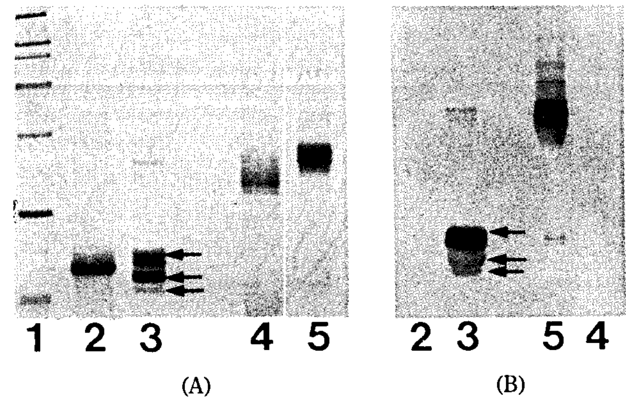

Fig. 2. SDS-PAGE (A) and immunoblot (B) analyses of HLTSA. 2 μg of purified HLTSA and HRTSA electrophoresed in 12% reducing (lane A-2 for HLTSA, lane A-3 for HRTSA) and nonreducing (lane A-4 for HLTSA, lane A-5 for HRTSA) SDS-PAGE and their corresponding immunoblot (B). Upper arrow indicates the 25-kDa intact form of HRTSA; lower two arrows, the fragmented HRTSAs. Lane 1 is size marker. The molecular masses, from the bottoms, 21.5, 31, 45, 66.2, 97.4, 116.25, and 200-kDa.

		P1 P2 P3		(Identity)
HLTSA	1	GLFIIDPNGVIKHLSVNDLPVGRSVEETLR	30	
MMER5	186	GLFIIDPNGVIKHLSVNDLPVGRSVEETLR	215	(100%)
BSP22	185	GLFIIDPNGVIKHLSVNDLPVGRSVEETLR	214	(100%)
HMERL	47	GLFIIDPNGVIKHLSVNDLPVGRSVEETLR	76	(100%)
HPAG	129	GLFIIDDKGILRQITVNDLPVGRSVDETLR	158	(70%)
HBTSA	128	GLFIIDGKGVLRQITVNDLPVGRSVDEALR	157	(70%)
NKEFB	128	GLFIIDGKGVLRQITVNDLPVGRSVDEALR	157	(70%)
NKEFA	129	GLFIIDDKGILRQITVNDPPCCRSVDETLR	158	(60%)

Fig. 3. Comparision of amino acid sequences of tryptic peptides from **HLTSA** with those of other TSA-like proteins. The deduced amino acid sequences of TSA-like proteins are listed along the following sequences: **MMER5** (*Mer5* gene that is expressed in murine erythroleukemia cell line, the mer5 gene product), **BSP22** (bovine mitochondrial SP22 protein precursor), **HMERL** (human Mer5-like gene product), **HPAG** (human mammary epithelial cell line HBL100, *PAG* gene product), **HBTSA** (human brain, hippocampus, TSA, HPRP gene product), **NKEFB** and **NKEFA** (human erythroleukemic cell line K562, natuaral killer cell enhancing fators B and A, respectively). The deduced amino acid residues are numbered (right and left margins) begining with the residue immediately after the initiating methionine, but the determined amino acid sequence of HLTSA is arbitrarily numbered.

In order to compare the structural property of HLTSA with the other known TSA isoforms, partial amino acid sequences of tryptic peptides were carried out. Three pure tryptic peptides of the HLTSA separated from the C_{18}-column, P1, P2, and P3 with retention times of 69.19, 55, and 49.89 min respectively showed amino acid sequences of P1: GLFIIDPNGVIK, P2: HLSVNDLPVGR, and P3: SVEETLR. amino acid sequences of the well isolated three peptides (P1, P2, and P3) were determined. Their retention times were

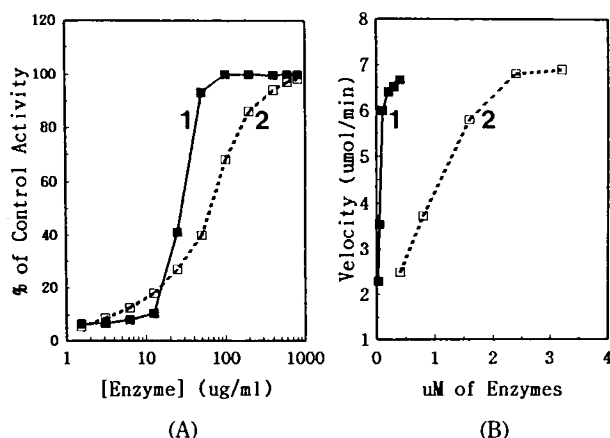

Fig. 4. Comparisions of protections of glutamine synthetase by HLTSA (curve 2 in figure A) and HRTSA (curve 1 figure A), and initial velocities of NADPH oxidations by HLTSA (curve 2 in figure B) and HRTSA (curve 1 in figure B). (A): various amount of HLTSA and HRTSA were added into the inactivation mixture (50 μl) containing 5 μg of *E. coli* glutamine synthetase, 10 mM DTT, 3 μM $FeCl_3$, and 50 mM Hepes-NaOH, pH 7.0. After 60 min inactivation at 37℃, 8 μl of sample was assayed for the remaining activity. (B): the reaction mixture (300 μl) containing 50 mM Hepes-NaOH, pH 7.0, 12.5 μg/ml of TR and Trx, 0.25 mM NADPH, 50 μM H_2O_2, and varying concentrations of HLTSA or HRTSA. The decrease in NADPH at 25℃ was mesured at 340 nm.

69.19 (P1), 55 (P2), 49.89 min (P3). An analysis of the homology search in the data base (BLASTP 1.4.8 M) showed that P1, P2, and P3 are linked in sequence by their designated peptide number and were found to retain 100% homology with the murine erythroleukemia cell Mer5 gene product (MMER), the bovine mitochondrial protease substrate protein, SP22 protein precursor (BSP22), and human Mer5-like protein (HMERL) (Fig. 3). The 30 residue peptide of HLTSA also showed a 70% sequence homology with the PAG gene product from human mamary epithelial cell line HBL100 (HPAG), and the natural killer cell enhancing factor A and B from human erythrolekemia cell line K562 (NKEFB and NKEFA). Thus, an identify of the partial amino acid sequence of HLTSA with other Mer 5-like human TSA suggests HLTSA as the third type of isoenzyme.

A comparision of the antioxidant activities of HLTSA and HRTSA

Fig. 4-A shows the TSA concentration-dependent protection activity against the inactivation of GS by the DTT/Fe^{3+}-catalyzed oxidation (thiol MCO) system. The inactivation was completely prevented by HLTSA (curve 2 in Fig. 4-A) or HRTSA (curve 1 in Fig. 4-A). HLTSA showed a slightly lower extent of GS protection than HRTSA. When thiol is replaced with another electron donor (e.g. ascorbate), HLTSA no longer protects against MCO system-induced GS inactivation (data not shown).

We have searched for an enzyme or an *in vivo* thiol-reducing equivalent capable of supporting the antioxidant activity of HLTSA against the ascorbate MCO system. Glutathione (GSH), known as an *in vivo* thiol reducing equivalent was monitored for its ability to give HLTSA potential for preventing the inactivation of glutamine synthetase by the ascorbate MCO system. Excess GSH was required to restore the antioxidant activity of HLTSA. Below 10 mM of GSH, HLTSA did not show a superior antioxidant activity to GSH itself (data not shown).

Trx-linked peroxidase activity of HLTSA

We previously reported the Trx-linked peroxidase activity of HRPRP (Cha *et al.*, 1995). Therefore, we examined the peroxidase activity of HLTSA. The peroxidase activity was indirectly measured by monitoring the oxidation of NADPH in the presence of *E. coli* Trx and Trx reductase (TR). The velocity of NADPH oxidation decreased with time showing a characteristic first order kinetics toward substrate, and the decrease was more overcome as the concentration of H_2O_2 increased (data not shown). At equivalent concentrations of H_2O_2 or alkyl hydroperoxides, 50 μM, the initial rates of the oxidations were increased as a function of the concentration of HLTSA (curve 2 in Fig. 4-B for H_2O_2). The increase in rate as a function of the concentration of HLTSA was much slower than that of HRTSA. The rates of NADPH oxidations by HLTSA (0.91, 1.08, and 1.16 μmol/min/nmol of HLTSA in the presence of H_2O_2, t-butyl hydroperoxide, and cumene hydroperoxide as a substrate, respectively) were negligible as compared to the rates of HRTSA (16.4, 10.51, and 10.42 mol/min/nmol of HRTSA with H_2O_2, t-butyl hydroperoxide, and cumene hydroperoxide, respectively). This result reduces the possibility of Trx as the *in vivo* thiol reducing equivalent.

We previously reported the abundant existence of a 25-kDa protein (HRPRP or HRTSA) in human RBC (Lim *et al.*, 1994) and its classification as the same protein as human brain TSA or NKEFB (Cha *et al.*, 1995). In this communication, we report the existence of a new type of TSA in human liver. Its thiol-dependent activity and a molecular mass of dimer in nonreducing SDS-PAGE suggest that HLTSA may be one member of the human TSA family. Analyses of tryptic peptide sequences of HLTSA and its inactivity toward the antibody of the yeast TSA indicate that HLTSA may be a Mer5-like type of human TSA.

An analysis of data base revealed a lot of protein sequences showing a very similar homology to TSA.

As a member of the TSA family, AhpC found in *Salmonella tyohimurium* and *E. coli* showed a peroxidase activity linked to F52 flavo protein in the absence of Trx (Jacobson *et al.*, 1989). We reported that a new type of Trx-linked thiol peroxidase from *E. coli* (Cha *et al.*, 1995) is a different protein from AhpC and that unlike this protein, AhpC did not exert a peroxidase activity linked to Trx (Cha *et al.*, 1995). Therefore, it is notable that unlike HRTSA, HLTSA did not show any significant peroxidase activity linked to Trx. The poor coupling of HLTSA to Trx gave a hint that Mer5-like HLTSA may be a eucaryotic counterpart of procaryotic AhpC. To investigate this possibility, we are searching for a F52-like protein in mammalian liver tissue.

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