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Purification of Sulfhydryl Oxidase from Human Foreskin Tissue and Immunohistochemical Localization†

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= Abstract = **Human sulfhydryl oxidase, catalyzing the conversion of either free or bound thiol to disulfide compound, was isolated from human skin tissue to apparent homogeneity through multiple steps of ammonium sulfate salting-out, DEAE-cellulose chromatography, CM-cellulose chromatography and ACA54 gel filtration. The enzyme was shown to have a molecular weight of 65 kDa and a specific activity of 8.39 x 10^3 U/mg protein. The specific polyclonal antibody was raised, with which the tissue distribution of the enzyme was studied immunohistochemically. The enzyme is present ubiquitously in most human tissues. However, the granular layer of the epidermis, stromal tissues of the breast and uterine cervix, hepatocytes and islets of the pancreas are noted to contain a comparatively high amount of the enzyme.**

Key Words: *Sulfhydryl oxidase, Human skin, Tissue distribution*

INTRODUCTION

It is well known that the thiol/disulfide status of the cell plays the critical role in regulation of metabolism and cellular function such as maintenance of intracellular redox potential, detoxifying capacity, metabolic control and conformational status of proteins. In the regulation of thiol/disulfide status, the role of sulfhydryl oxidase is important. The enzyme catalyzes the aerobic oxidation of sulfhydryl groups to disulfide bonds as follows.

$$2RSH + O_2 \longrightarrow RSSR + H_2O_2$$

In the above equation, RSH and RSSR represent the reduced and oxidized forms of either small thiol containing substances, such as glutathione or thiol containing large molecules, such as reductively denatured chymotrypsinogen A, xanthine dehydrogenase or pancreatic RNAse A etc.

The sulfhydryl oxidase has been purified from a variety of sources such as bovine milk (Claire *et al.* 1984; Janolino *et al.* 1975), cow skin (Yamada *et al.* 1987), rat kidney (Ormstad *et al.* 1979), and bovine pancreas (Claire *et al.*

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1988) etc. With the enzymes, some kinetic studies and physiological experiments have been carried out, for example, on its involvement in the keratinization process (Ogawa *et al.* 1979), and in the development of motility during sperm epididymal maturation (Cornwall *et al.* 1988) etc. Despite the many studies, there has been little systemic analysis of the enzyme except for biochemical activity monitoring in tissue (Claire *et al.* 1984).

Therefore, it would be interesting to analyze systemically the tissue distribution of the enzyme and to compare its biological roles in tissue, especially in human tissue.

METHODS AND MATERIALS

Reagents

Dithiothreitol (DTT), reduced glutathione (GSH), Tris (hydroxymethyl) aminomethane, ethylenediaminetetraacetate (EDTA) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Co. (Monsanto. USA). DEAE-cellulose, CM-cellulose, and other column chromatography reagents were obtained from Pharmacia Fine Chemicals. Other reagents of analytical grade were purchased from the available local commercial sources.

Preparation of human skin samples

The human foreskin tissue was collected at Seoul National University Hospital immediately after circumcision operation. The skin tissues were stored at -70°C in a deep freezer until analysis after twice washing out blood with physiological saline.

Assay for sulfhydryl oxidase

Enzyme activity was assayed by monitoring the ability of enzymatically active preparations to oxidize Ellman's reagent (Takamori *et al.* 1980). To describe the method briefly, a typical reaction mixture contained 0.1 mM DTT substrate, 0.1 ml enzyme mixture and diluent buffer to make a total volume of 1.2 ml. The mixture was incubated at 37°C, and aliquots of 0.3 ml were removed at 0, 30 and 60 min and added to 3.0 ml diluent buffer. Fifty μl of DTNB was added and absorbance at 412 nm was measured. Using the molar absorbance of DTNB (1.3×10^4 at 412 nm), the data were expressed as nanomoles of oxidized DTNB per hour per microgram protein.

Purification of the enzyme

The enzyme was purified through the fractional salting-out process with ammonium sulfate of 70% and 95% saturation, column chromatography through DEAE-cellulose and CM-cellulose, followed by ACA54-gel filtration. The frozen human foreskin tissue was pulverized in liquid nitrogen, followed by homogenization in Tris-acetate buffer (pH 7.6, 0.1 M) with polytron homogenizer. The homogenate was centrifuged for 30 minutes at 6,000 x g in the cold room to eliminate unruptured cells and large cell debris. The supernatant of the sample was subjected to salting-out with ammonium sulfate at 95% saturation. After dialysis overnight against Tris-acetate buffer (0.05 M, pH 7.6), the samples were centrifuged down for one hour at 30,000 x g. The supernatants were subjected to DEAE-cellulose chromatography, which was eluted with NaCl gradients (0-1 M). The active fractions were collected and concentrated by the centricon method. The concentrated samples after dialysis were applied to CM-cellulose column chromatography with NaCl gradients (0 M-0.5 M). The active fractions were pooled and concentrated again. And the concentrates were subjected to ACA54 gel filtration. The active fractions were pooled and analyzed for enzymic activity and protein pattern.

Preparation of specific antibody

A specific antibody to the human foreskin sulfhydryl oxidase was raised in rabbits via multiple injection of the sample (30 μg each time), twice with Freunds complete adjuvants and twice again with incomplete Freunds adjuvants at two week intervals each time. The raised antibody was confirmed by Ouchterlony's method as well as by Western blot analysis.

Immunohistochemical analysis

Various tissue samples from normal human beings, fixed in formalin and embedded in paraffin, were collected at the Department of Pathology, Seoul National University Hospital. The samples were subjected to immunohistochemical analysis with successive treatment of primary antibody to sulfhydryl oxidase, biotinylated secondary antibody and avidin-biotin-peroxidase complex, followed by visualization with diaminobenzidine peroxidation.

RESULTS

Purification of sulfhydryl oxidase from human foreskin tissue

The purification data for the sulfhydryl oxidase were summarized as shown in Table 1. The enzyme from the human foreskin tissue was purified almost to homogeneity as shown in Tabl 1, with 32% yield and 252 fold purification. In the DEAE-cellulose chromatography, the enzymic activities were eluted without salt gradient, which suggests its weak binding to anionic exchange column. In the CM-cellulose chromatography, the enzymic activities were eluted separately at low salt fractions(0.05 - 0.1 M) and high salt fractions (NaCl 0.5 - 0.8 M NaCl)(Fig. 1). Between fractions, since low salt-eluted active fractions showed higher specific activity, the fractions were pooled and concentrated for the next step(Fig. 1). In the

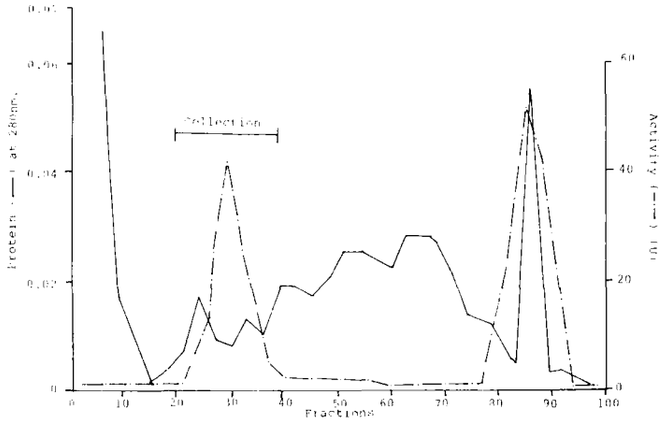

Fig. 1. Elution profile of human skin sulfhydryl oxidase in carboxymethyl cellulose chromatography.

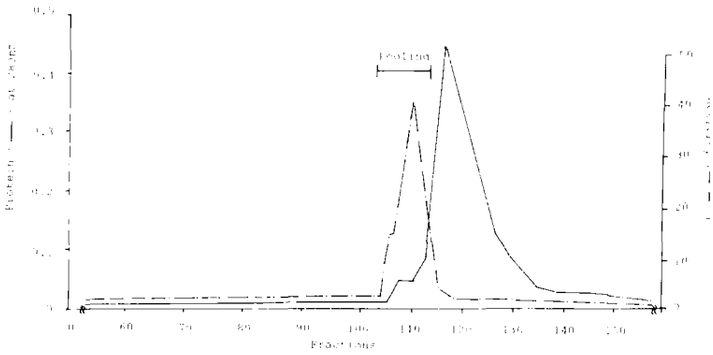

Fig. 2. Elution profile of human skin sulfhydryl oxidase in ACA54 gel filtration column.

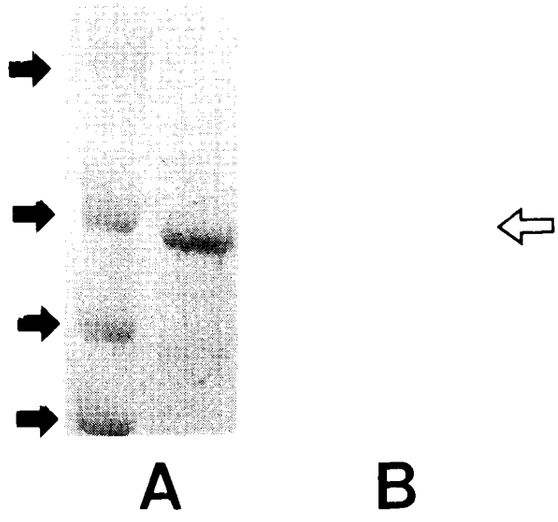

Fig. 3. Purified sulfhydryl oxidase and western blot analysis.
(A) SDS-PAGE analysis of purified sulfhydryl oxidase. Left lane: molecular weight standards of 116 kDa, 67 kDa, 45 kDa and 29 kDa from above. Right lane: purified human foreskin sulfhydryl oxidase
(b) Western blot analysis of human skin homogenates (left lane) and isolated sulfhydryl oxidase (right lane) with anti-sulfhydryl oxdase antibody.

ACA54 gel filtration, the enzymic active fractions were pooled and concentrated for analysis(Fig. 2). When the isolated sulfhydryl oxidase was subjected to SDS-PAGE, the active enzymic protein was shown to have a molecular weight of 65 kDa(Fig. 3), which was used for the antibody preparation. The antibody toward the enzyme

Table 1. Purification table of skin sulfhydryl oxidase

Purification step	Volume (ml)	Total protein (mg)	Total enzyme activity (U)	Specific activity(U/mg)	Purification fold	Yield (%)
Homogenate	712	2,300	525,783.4			
95% Salt-out	70	892.5	29,732.0	33.3	1.0	100
CM-cellulose	135	54.0	21,600.0	400.0	12.0	72.7
ACA 54 gel	1.5	0.825	6,922.5	8,390.9	251.9	32.0

was confirmed to react with the enzyme specifically as shown in the western blot analysis of human skin tissue homogenate(Fig. 3).

Distribution of sulfhydryl oxidase in human tissues

As summarized in Table 2, the immunohistochemical study showed the ubiquitous presence of sulfhydryl oxidase in human tissue. In which a higher concentration of the enzyme was noted in the epidermal granular layer of skin, stroma of the testes, breast, uterine cervix, hepatocyte and islets of pancreas, etc. The prominent presence of this enzyme in most of the stromal tissue was marked. In the cases of muscle tissue, the heart muscle was enriched with the enzyme, while smooth muscles had a low amount of the enzyme, and the skeletal muscle had more enzymes than the smooth muscle.

In the skin tissue, a higher concentration of sulfhydryl oxidase was observed(Fig. 4), though other accessory organs were also positively reacted. In the breast tissue, the stroma and nipple epithelial cells were highly enriched

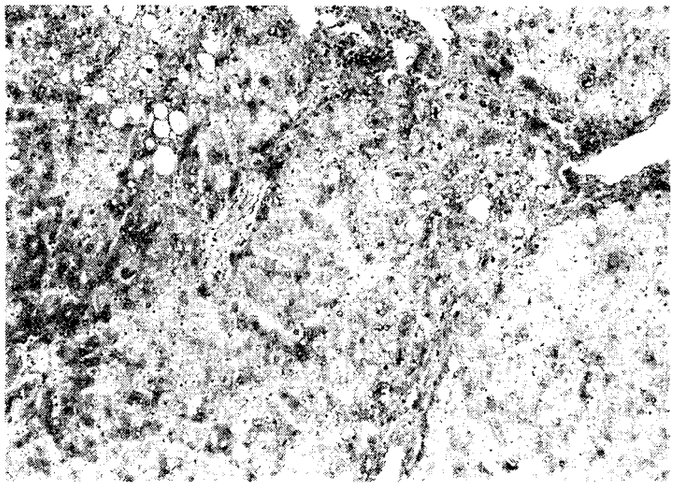

Fig. 5. Immunohistochemical pattern of human liver reacted to anti-human skin sulfhydryl oxidase antibody.

Fig. 4. Immunohistochemical pattern of human skin tissue reacted to anti-human skin sulfhydryl oxidase antibody.

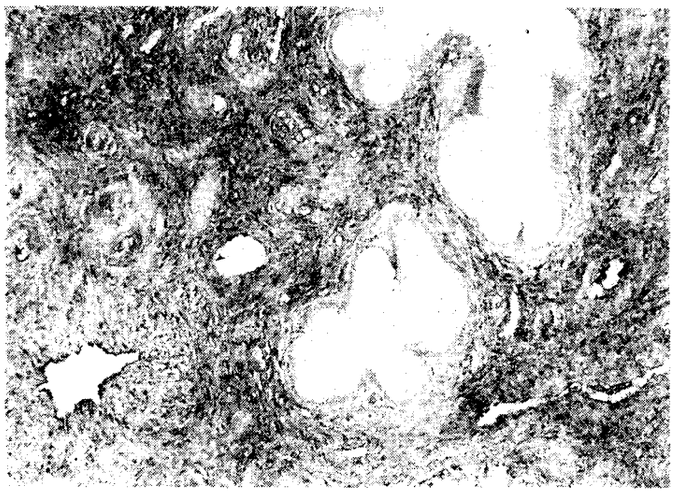

Fig. 6. Immunohistochemical pattern of human uterine tissue reacted to anti-human skin sulfhydryl oxidase antibody.

with the enzyme. In the gastrointestinal tracts, the intestinal epithelial cells usually had a very small amount of the enzyme, while the submucosa tissue was enriched. The liver tissue showed a relatively high amount of the enzyme (Fig. 5). In the genitourinary system, most of the kidney tissue showed strong reactivity to the anti-sulfhydryl oxidase antibody. And the stroma of the uterus showed a considerable amount of the enzyme(Fig. 6). Also in the testes, the stroma was positive, while Leydig cells and spermatocytic cells did not respond strongly to the antibody(Fig. 7). In addition, a considerably strong immunological response was observed diffusely in the cerebral tissue and the parathyroid gland.

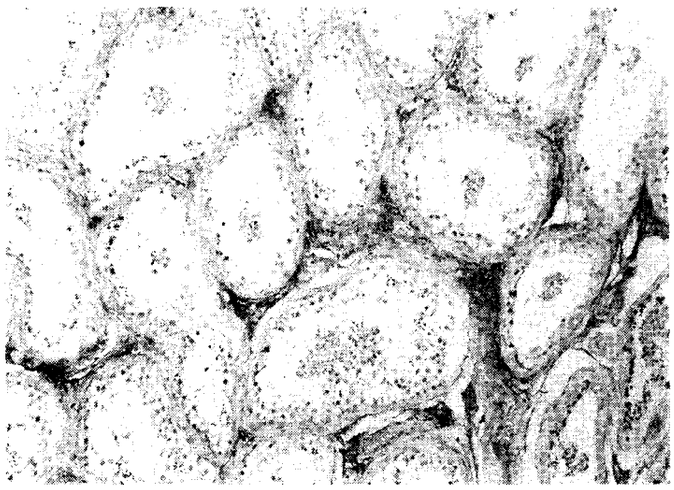

Fig. 7. Immunohistochemical pattern of human testes reacted to anti-human skin sulfhydryl oxidase antibody.

Table 2. Immunohistochemical analysis of tissue distribution of sulfhydryl oxidase in human tissue

Tissue	Immunological reactivity to sulfhydryl oxidase antibody
Cutaneous tissue	
skin	
epidermis	+ +
dermis	+
blood vessel	+
sweat gland	+
breast	
duct/acini	±
myoepithelial cell	+
stroma	+ +
nipple	
epithelial cells	+ +
sebaceous gland	+ +
subcutaneous	+
Gastrointestinal tract	
salivary gland	
acini	−
stroma	+
duct epithelium	±
esophagus	
squamous epithelial cell	+
submucosal cell	+
smooth muscle	−
peripheral nerve	±
stomach	
chief cell	−
parietal cell	±
foveolar epithelium	−
mucosal muscle	±
submucosa	+
muscle	−
nerve	±
serosa	+
small intestine (duodenum & ileum)	
villous epithelium	−
crypt	−
lamina propria	+
submucosa	+
smooth muscle	±
nerve	±
large intestine	
absorptive epithelium	−
crypt	−
lamina propria	+
submucosa	+
smooth muscle	±
nerve	±
serosa	+
liver	
hepatocyte	+ +
bile duct	±
pancreas	
acinus	+
islets	+ +
ducts	±
gall bladder	
epithelium	+
submucosa	+
muscle	±

Genitourinary system	
kidney	
glomeruli	+
proximal tubule	+
distal tubule	+
urinary bladder	
transitional epithelium	—
submucosa	+
smooth muscle	±
prostate	
epithelium	+
smooth muscle	±
adrenal gland	
zona glomerulosa	—
zona reticularis	—
zona fasciculata	—
medulla	—
ovary	
stroma	+
granulosa cell	—
theca cell	—
testes	
tubule	±
Leydig cell	±
stroma	+
uterus	
endometrial gland	±
endometrial stroma	+ +
myometrium	±
placenta	
trophoblast	—
stroma	±
blood vessel	+
Other tissues	
cerebrum	+
parathyroid gland	+
skeletal muscle	+
thyroid	
thyrocyte	±
colloid	—
heart muscle	+
red blood cell	+
eosinophil	+
neutrophil	—
lymphocytes	—

+ + strong positive reaction to anti-human skin sulfhydryl oxidase antibody.
+ positive reaction
± weak positive reaction
— negative reaction

DISCUSSION

Maintaining the thiol/disulfide ratio is important in intracellular redox potential, detoxification, metabolic regulation and protein folding. In the case of protein folding, though thermodynamically favored, the rate of spontaneous oxidation of sulfhydryl group to form disulfide bond is too slow to explain the intracellular generation of those bonds. However, the mechanism which generates and maintains the significant levels of disulfide bonds in the mammalian cells is not clearly known. Up to now, several different types of enzymes have been described to be involved in extracellular thiol oxidation, such as flavin-dependent oxidase in seminal vesicle secretion, a copper-containing thiol oxidase in the basal lateral region of the kidney and intestine, and an iron-dependent sulfhydryl oxidase in bovine milk and kidney(Ormstad *et al.* 1979 & 1981; Ostrowski *et al.* 1979; Ostrowski & Kistler 1980). Recently, sulfhydryl oxidase was purified and characterized from bovine and murine skin tissues(Janolino & Swaisgood 1975; Takamori *et al.* 1980). In addition to sulfhydryl oxidase enzymes, the disulfide bond formation can be catalyzed by transhydrogenation reaction or peroxidase catalyzed reaction or free radical reactions(Ormstad *et al.* 1981). Therefore, it is not easy to assess the mechanism underlying disulfide bond formation. However, various biological functions are deeply involved with covalent disulfide bond formation.

In the keratinization process of the skin tissue, the crosslinking reactions of the keratins or other matrix components of the cornified envelopes by transglutamination or disulfide bond formation play the paramount important roles. Actually, the disulfide bond formation was shown to occur at the junction of the living and horny layer of the epidermis(Ogawa *et al.* 1979). And biochemical and histochemical analysis revealed considerably higher activity in the epidermis, while the dermis also contained some activity, of which the molecular weight was

found to be 65 kDa (Yamada *et al.* 1987; Takamori *et al.* 1980).

High sulfhydryl oxidase activity has been found and purified, in bovine milk as well as in the milk from other animals such as goats, pigs, rats and also in humans (Clare *et al.* 1984). Immunological study showed that a similar enzyme is present in pancreatic acinar cells and kidney cortex which was a membrane-associated glycoprotein with molecular weight of 300 kDa as an aggregated form of 90 kDa subunit(Schmelzer *et al.* 1985).

In purification of sulfhydryl oxidase from kidney tissue, copurification of glutathione oxidase activity with γ-glutamyl transpeptidase activity hampered its characterization. However, covalent chromatography on cysteinyl succinamido propyl-derivatized column, gel permeation on Biogel P-200 and immunological analysis could separate the two enzymes (Schmelzer *et al.* 1984 & 1985; Sliwkowski *et al.* 1983). The multiplicity of the enzyme and the analogous reactions of other enzymes such as transhydrogenation and peroxidation have confused the study of sulfhydryl oxidase.

In human, there have been few studies on sulfhydryl oxidase except for some works in relation to human skin(Takamori *et al.* 1980). However, most of the research in relation to sulfhydryl oxidase has been limited to the biochemical analysis of its activities.

In the present experiment, we have purified sulfhydryl oxidase from human foreskin tissue near to homogeneity(Fig. 3). The purified enzyme was essentially very similar to previously-reported rat skin enzyme in its biochemical properties of enzyme purification and in molecular size of 65 kDa (Takamori *et al.* 1980). In this study, the sulfhydryl oxidase of the human skin tissues was not bound to DEAE-cellulose anionic exchange column, and the finally purified enzyme showed its molecular weight to be 65 kDa similar to other skin enzymes from rats and cow snout(Takamori *et al.* 1980; Yamada 1987). The SDS-PAGE analysis showed that it was purified to near homogeneity (Fig. 3), and the western blot analysis indicated the specificity of the enzyme, since the antibody reacted to the only single protein band of 65 kDa size of the total skin homogenate (Fig. 3). Therefore, with this sulfhydryl oxidase specific antibody, it would be pertinent to continue our immunohistochemical study.

Previous study has illustrated the high content of the sulfhydryl oxidase in the granular layer of the skin tissue by activity monitoring (Yamada *et al.* 1987). In our study with prepared sulfhydryl oxidase-specific antibody, we could confirm its high concentration in the granular layer (Fig. 4). The enzyme was densely present in the granular layer and also considerably present in the dermis.

The activity monitoring of the tissue distribution showed high activities in mammary glands, kidney and pancreas, while other tissue such as thymus, heart, brain, liver, spleen, lung and small intestines showed negligible activities(Clare *et al.* 1984). However, in our immunohistochemical study of human tissue with anti-human skin sulfhydryl oxidase antibody, the enzyme was found to be ubiquitously present, though wide differences in its content could be observed. As summarized in Table 2, the markedly high content of the enzyme was noted in the tissue of the epidermis, stroma of the breast, uterine cervix, islets of pancreas and hepatocytes(Fig. 5,6).

It is interesting that the stromal tissue is enriched with sulfhydryl oxidase enzymes, probably because the crosslinking reactions of disulfide bond formation would be required to maintain the stability of the connective tissues. An earlier study indicated its high content in the pancreas(Clare *et al.* 1984 & 1988) which was compatible with our study, indicating its higher content in the islets of the pancreas. However, it is not clear whether the previously reported pancreatic sulfhydryl oxidase is identical to the skin sulfhydryl oxidase of the present study or simply immunologically cross-reactive or a totally different enzyme. The high content of anti-sulfhydryl oxidase antibody-reactive substances in the hepatocytes attracted our special concern. Since the activity monitoring did not

reveal a high content of the enzyme in the liver tissue(Clare *et al.* 1984) contrary to our study, it would be pertinent to study the expression and processing of the enzyme in liver tissue.

From these results, it can be summarized that human skin sulfhydryl oxidase has a molecular weight of 65 kDa, which is present ubiquitously in most tissue, with high amounts in the granular layer of the epidermis, stroma of the breast and uterine cervix, hepatocyte and pancreatic islet cells. The biological role of the enzyme in different tissue would reveal the interesting regulation mechanism in maintenance of the thiol/disulfide ratio for metabolic and cellular regulation, functional ability of proteins in association with conformation adjustment and other biological process including keratinization, sperm motility etc.

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