Novel 28-kDa secretory protein from rat olfactory epithelium

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Abstract We have isolated a novel secretory 28-kDa protein which is an abundant component of the rat olfactory mucosa. The partial sequence of the 28-kDa protein has been determined. The amino acid sequence of the 28-kDa protein is similar to that of non-selenium glutathione peroxidase from bovine ciliary body. The 28-kDa protein catalyzed decomposition of the hydrogen peroxide as well as organic hydroperoxides by reduced glutathion and seems to be a member of the glutathion peroxidases family.

Key words: 28-kDa protein; Glutathione; Glutathione peroxidase; Olfactory epithelium; Rat

1. Introduction

Although considerable progress was made in understanding the molecular mechanisms of odorant recognition and signal transduction [1,2], relatively little is known about the perireception processes. These perireceptor events occur primarily in the mucus layer covering the olfactory mucosa. Mucus consists of a variable admixture of water, electrolytes and proteins secreted largely by sustentacular cells and Bowman's glands [3]. Recently a novel specific water-soluble 45-kDa GTP-binding protein was found in the rat olfactory epithelium [4,5]. The high amount of 45-kDa protein suggests an important role of these proteins in perireceptor events. Another major protein which is present in equal amount in rat olfactory mucus is 28-kDa protein. In the present paper we describe some properties and determination of the amino acid sequence of this 28-kDa secretory protein.

2. Materials and methods

2.1. Materials

[^{35}S]Methionine was obtained from the Physics and Energy Institute, Obninsk, Russia; peroxidase-conjugated goat anti-rabbit Ig, Hybond C membrane from Amersham, UK; Triton X-100, 3′,3-diaminobenzidine, Dulbecco's modified Eagle's Medium (DME), reduced glutathione (GSH), glutathione reductase (GSSG-reductase), cumene hydroperoxide, NADPH, and chemicals for electrophoresis from Sigma (St. Louis, USA); DEAE-Sepharose and Sephacryl S-200 from Pharmacia (Uppsala, Sweden).

2.2. Isolation of 28-kDa protein

The 28-kDa protein was isolated as described earlier with modifications [5]. Briefly, isolated rat olfactory mucosa was homogenized in Dulbecco's modified Eagle's Medium (DME), pH 8.0 (1 ml per mucosa). The homogenate was centrifuged and supernatant was dialyzed overnight against buffer, consisting of 12 mM Tris-HCl at pH 7.8, 1 mM $MgCl_2$, 1 mM dithiothreitol. The dialyzed extract was applied to a DEAE-Sepharose column and the column was eluted with a 750-ml gradient of 0–500 mM NaCl in the same buffer. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing the 28-kDa protein were concentrated and applied to a Sephacryl S-200 column equilibriated with buffer, consisting of 25 mM Tris-HCl at pH 7.8, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol. Fractions containing purified 28-kDa protein were collected.

2.3. Production of polyclonal antiserum against 28-kDa protein

A female New Zealand white rabbit was immunized in the back with 400 μg of 28-kDa protein in complete Freund's adjuvant. The immunization procedure was repeated 4 weeks later with the same amount of antigen emulsified in incomplete Freund's adjuvant. Antiserum was obtained 10 weeks after the second injection.

SDS-polyacrylamide gel electrophoresis was performed in the discontinues buffer system of Laemmli [6]. Immunoblotting was performed on Hybond C membrane as described [4].

2.5. Synthesis of 28-kDa protein in intact olfactory epithelium

Olfactory mucosa was isolated and washed in Hank's Medium at room temperature. Washed tissue was incubated for 1 h at 37°C with [^{35}S]L-methionine (1 μCi/μl). Immediately after the incubation, tissue was homogenized in saline and centrifuged at 20,000 × *g*. The supernatant and incubation medium were analyzed by SDS-electrophoresis followed by autoradiography. Stimulation of the mucosa was performed by addition of chloroform (10 μl) to 2 ml of the medium.

2.6. Partial sequencing of 28-kDa protein

Purified 28-kDa protein was reduced and alkylated essentially as described [7]. The modified 28-kDa protein was digested by lysyl-endopeptidase and the peptides were separated by HPLC. The amino acid sequences were analyzed by automatic Edman degradation using a Applied Biosystem 470A gas phase sequenator.

2.7. Enzyme assay

GSH Px activity was measured spectrophotometrically by a modification of the procedure described by Shichi and Demar [8]. The standard assay mixture (2.0 ml) contained: 50 mM Tris-HCl pH 7.2, 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 mM GSH, 1 U GSSG-reductase, 0.25 mM H_2O_2 or 1.5 mM cumene hydroperoxide.

Protein concentrations were measured according to the method of Bradford [9] using bovine serum albumin as a standard.

3. Results and discussion

Fig. 1a shows a SDS-electrophoresis pattern of water-soluble extract of rat olfactory epithelium. Only several major bands are revealed in gel including a 45-kDa protein which was described earlier [4,5]. The next major band corresponds to a 28-kDa protein which is another abundant component of water-soluble extract. We have isolated this protein in preparative amount by DEAE-chromatography and gel-filtration (Fig. 1a). The molecular mass of the protein estimated by gel-filtration was about 60 kDa. These results indicate that the protein is composed of two identical subunits of about 28-kDa.

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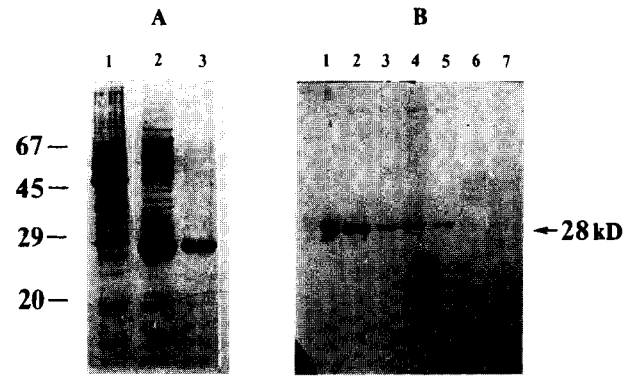

Fig. 1. (A) SDS-electrophoresis patterns of the 28-kDa protein at different steps of purification. Samples were run in 10% polyacrylamide gel; (1) isotonic extract of rat olfactory epithelium; (2) DEAE-Sepharose peak; (3) Sephacryl S-200 peak. Coomassie R-250 staining. (B) Immunoblots of purified 28-kDa protein and water-soluble proteins of different rat tissues. Visualization by rabbit antibodies against 28-kDa protein. (1) Purified 28-kDa protein; (2) isotonic extract of rat olfactory epithelium; (3–7) hypotonic extracts obtained from liver (3), lung (4), kidney (5), skeleton muscle (6), and brain (7). Samples were run in 10% polyacrylamide gel.

Approximately 15–20 μg of purified 28-kDa protein was routinely obtained from one rat mucosa. We estimate that 28-kDa protein may represent up to 3% of the total water-soluble proteins.

The rabbit polyclonal antibodies raised against the native 28-kDa protein have been obtained (Fig. 1b). Immunoblots with this antibody revealed a single 28-kDa band immunoreactivity in pattern of rat olfactory epithelium and less potent reactivity in lung (Fig. 1b). We detected very weak or no reactivity in other rat tissues (liver, brain, muscle, kidney, intestine). Some attempts were made to reveal this protein in other species. We examined olfactory epithelium of non-mammals, frog and fish, using the antibodies against the 28-kDa, but could not detect any analogies of the 28-kDa protein.

To determine the localization of the 28-kDa protein we used immunohistochemical studies at both the light microscopic and electron microscopic level. Our observation shows that the 28-kDa protein is localized in the secretory granules of sustentacular cells and in the lower level of the mucus, surrounding olfactory cilia, dentritic knobs, and microvilli of the sustentacular cells (manuscript in preparation). We also revealed the 28-kDa protein in the secretory granules of the trachea epithelium cells. To study the 28-kDa protein metabolism in olfactory epithelium we performed the experiments on [^{35}S]methionine uptake in isolated olfactory mucosa. Analysis of labeled proteins by SDS-electrophoresis and autoradiography indicated that [^{35}S]methionine was mainly incorporated into 45-kDa and 28-kDa proteins (Fig. 2). An essential amount of these proteins was also revealed in incubation media. Moreover, the release of 45-kDa and 28-kDa proteins into incubation was stimulated by chloroform. Chloroform was chosen because it stimulates the secretory granules release from the olfactory cells [10]. Thus, combined data obtained from immunohistochemistry and metabolism studies suggest that the 28-kDa protein is a secretory protein.

To determine the possible function of the 28-kDa protein we made the partial sequence analysis of this protein followed by homology searches through the database. We have determined the N-terminal amino acid sequence of the protein (5 residues) and the sequence of three internal peptide fragments isolated after protein digestion with lysylendopeptidase (Fig. 3). Search through the database revealed the protein which contained all four determined sequences (Nomura, N., Miyajima, N., Kawarabayashi, Y. and Tabata, S. (1993) unpublished data, Gene Bank, Accession number D14662). The function of this protein is unknown but its N-terminal sequence has a high homology with that of the non-selenium glutathion peroxidase from bovine cilliary body [8].

Glutathione peroxidase (GSH Px) catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, or lipid peroxides by reduced glutathione (GSH). The 28-kDa protein catalyzed decomposition of hydrogen peroxide as well as organic hydroperoxides by GSH. The specific activity was 1.9 U for H_2O_2 and 2.7 U for cumene HP, GSH was essential and could not be replaced by dithiothreitol. The specific activity of 28-kDa protein is somewhat lower than those of many Se-dependent GSH peroxidases previously reported [11]. On the other hand, substrate specificity and enzymatic activity of the 28-kDa protein are comparable with those of specific GSH peroxidase from bovine ciliary body [8].

Two types of GSH Px are generally recognized; selenium GSH Px and non-selenium GSH Px which are distinguished on the basis of their substrate specificity: Se-enzyme reacts with both H_2O_2 and organic hydroperoxides such as cumene HP, while non-Se enzyme is active only with organic hydroperoxides [12]. So, one could suggest that the 28-kDa protein may belong to the family of selenium-dependent GSH Px. Some properties of the 28-kDa protein (substrate specificity, enzymatic activity, molecular mass, and N-terminal sequence identity) are similar to those of GSH Px from ciliary body that does not seem to belong to either type of GSH peroxidases. It is likely that they both are the member of a distinct glutathione peroxidases family. Based on 28-kDa protein enzymatic activity

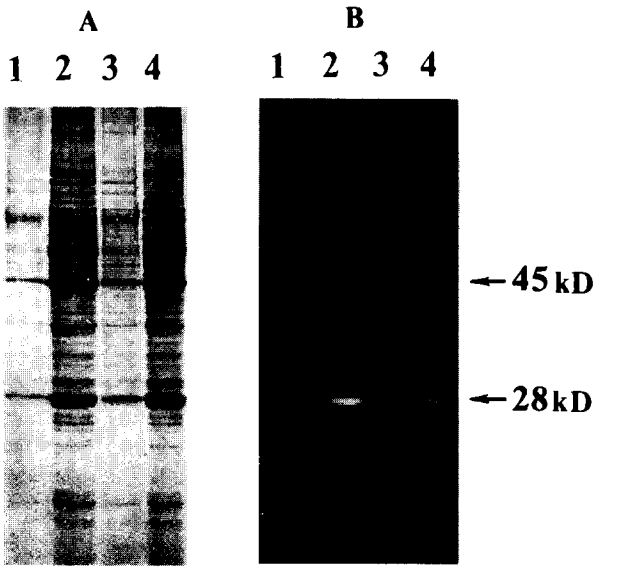

Fig. 2. Synthesis of the 28-kDa protein in intact olfactory epithelium. Tissue was incubated with [^{35}S]L-methionine (1 μCi/μl). SDS-electrophoresis patterns of incubation medium (1,3) and isotonic extract (2,4) were stained with Coomassie R-250 (A) followed by autoradiography (B). (1,2) control; (3,4) stimulation by chloroform.

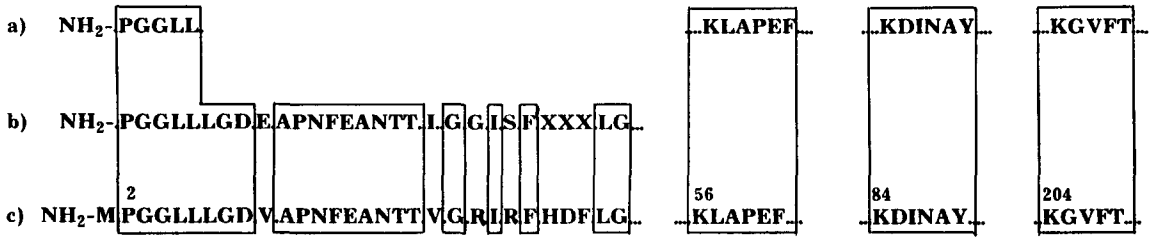

Fig. 3. Sequence analysis of the 28-kDa protein. (a) Sequence fragments of the 28-kDa protein; (b) N-terminal sequence of non-selenium glutathion peroxidase from bovine ciliary body [8]; (c) amino acid sequence of gene product from homo sapiens myeloblast cell, Gene Bank, Accession number D14662.

one of its proposed function may be the protection of the olfactory cells from oxidative damage resulting from lipid peroxidation. It is also possible that this protein functions in neutralizing peroxides in mucus.

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