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A Novel Selenium- and Copper-Containing Peptide with Both Superoxide Dismutase and Glutathione Peroxidase Activities

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Superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) play crucial roles in balancing the production and decomposition of reactive oxygen species (ROS) in living organisms. These enzymes act cooperatively and synergistically to scavenge ROS. In order to imitate the synergism of these enzymes, we designed and synthesized a novel 32-mer peptide (32P) on the basis of the previous 15-mer peptide with GPX activity and a 17-mer peptide with SOD activity. Upon the selenation and chelation of copper, the 32-mer peptide was converted to a new Se- and Cu-containing 32-mer peptide (Se-Cu-32P) that displayed both SOD and GPX activities, and its kinetics was studied. Moreover, the novel peptide was demonstrated to be able to better protect vero cells from the injury induced by the xanthine oxidase (XOD)/xanthine/Fe^{2+} damage system than its parents. Thus, this bifunctional enzyme imitated the synergism of SOD and GPX and could be a better candidate of therapeutic medicine.

Keywords: Superoxide dismutase, glutathione peroxidase, bifunctional enzyme, reactive oxygen species, synergism

Reactive oxygen species (ROS), mainly superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), are produced as by-products of mitochondrial respiration. There is a balance between the generation and elimination of ROS in living organisms [13]. If the balance is destroyed, excess ROS would be produced to oxidize lipids, protein, DNA, and carbohydrates, resulting in the breakdown of normal cellular function and generation of various diseases [1, 2, 19, 20].

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The human antioxidant enzyme defense system against ROS principally includes superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), and the antioxidant effect is carried out by sequential enzymatic reactions. In the first step of the process, SOD catalyzes dismutation of superoxide anions to H_2O_2. Then, GPX and CAT convert H_2O_2 to water independently [4]. However, any increase of SOD catalytic activity will produce excess H_2O_2, which must be neutralized timely by either GPX or CAT. Otherwise, the high concentrations of H_2O_2 will generate hydroxyl radicals through the Fenton or Haber–Weiss reaction. Then, the hydroxyl radical will rapidly react with unsaturated fatty acid side chains and result in lipid peroxidation and disruption of cell membranes [7, 8]. Thus, the balance between SOD and GPX or CAT activities is required for maintaining the normal ROS level and the normal function in living organisms.

There are some previous studies on the synergism of antioxidant enzymes. For example, SOD and CAT are expressed simultaneously to retard oxidative damage [15]. Superoxide dismutase and catalase are conjugated to polyethylene glycol (PEG) to increase endothelial enzyme activity and oxidant resistance [12]. Moreover, EUK, a synthetic mimic with SOD and CAT activities, is widely used to prevent oxidative stress [3, 10, 14]. Recently, we generated a bifunctional enzyme with both SOD and GPX activities by combination of fusion protein technology with the amino acid auxotrophic expression system [21]. However, this fusion protein is a kind of macromolecule and is expressed as inclusion body form, and thus, its application is restricted. Therefore, it is necessary to design a small peptide with both GPX and SOD activities to overcome the shortcomings of a macromolecule. In our previous studies, a SOD mimic, 17-mer copper–peptide (17CuP), was synthesized [11]. In addition, selenium-containing 15-mer

peptides (15SeP) with GPX activity were prepared [17]. In order to imitate the synergism of antioxidant enzymes, a novel 32-mer peptide was designed on the basis of the amino acid sequences of a previous 15-mer peptide and a 17-mer peptide, and synthesized by direct linkage of the 15-mer peptide with the 17-mer peptide to obtain a novel 32-mer peptide (32P). Upon the selenation and chelation of copper, the 32-mer peptide was converted to a Se- and Cu-containing 32-mer peptide (Se-Cu-32P) and displays both SOD and GPX activities. In the present paper, the preparation of the Se-Cu-32P and its characterization are described.

MATERIALS AND METHODS

Chemicals

BOP reagent, *N*-hydroxybenzotriazole (HOBt), thioanisole, *m*-cresol, 1,2-ethanedithiol (EDT), protected amino acids, and preloaded resins were purchased from GL Biochem (Shanghai). Phenylmethanesulfonyl fluoride (PMSF), glutathione reductase, β-nicotinamide adenine dinucleotide phosphate (NADPH), xanthine and xanthine oxidase (XOD), trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), and HPLC-grade acetonitrile were purchased from Sigma. All other regents and solvents were of analytical or higher grades.

Peptide Synthesis

The peptide was synthesized on a 0.1 mM scale from Fmoc-Ser (tBu)-Wang resin by Fmoc-based solid-phase peptide synthesis (SPPS). Side-chain protecting groups of amino acids included 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, *t*-butyl (tBu) for Tyr and Thr, trityl (Trt) for Asn and His, and *t*-butoxycarbonyl (Boc) for Trp. Preloading Wang resin was swollen in DMF (5 ml, 45 min). Fmoc deprotection for all amino acids was accomplished with 20% (v/v) piperidine/DMF (5 ml, 20 min). A 3-fold excess of the amino acids (0.3 mM) was activated with BOP reagent (0.3 mM), HOBt (0.3 mM), and *N*-methyl morpholine (NMM) (0.45 mM) and coupled to the resin. Each amino acid was coupled for 90 min or until ninhydrin test of the resin was negative.

Cleavage, Purification, and Analysis of Synthesized Peptide

Wang resin with the attached peptide was washed with DMF (6× 5 ml), methanol (6× 5 ml), and dried in a vacuum desiccator overnight. The dry resin was treated with Reagent K, which is consisted of TFA, m-cresol, EDT, thioanisole, and water, with a ratio of 82.5:5:5:5:2.5 (v/v/v/v/v), for 2 h. The solvent was filtered, and the resin was washed with TFA. The peptide was precipitated with cold diethyl ether. The pellet was isolated by centrifugation, collection, and purified at a semipreparative scale by reverse-phase HPLC and on a C-18 column (250×4.6 mm, 5 μm) with 220-nm UV detection. The column was equilibrated with solvent A (H_2O, 0.1% TFA), and then the peptide was eluted with a linear gradient of solvent A and solvent B (acetonitrile, 0.1% TFA). Target peptide was collected and its molecular weight was analyzed by MALDI–TOF–MS spectrometer.

Synthesis and Characterization of Bifunctional Peptide

Selenation of the 32-mer peptide was performed as described by the procedure of Wu and Hilvert [24]. Sodium hydroselenide was prepared according to the method of Klayman and Griffin [9]. Briefly, 1 mg of 32-mer peptide was dissolved in 1 ml of sodium phosphate buffer (50 mM, pH 7.0), 20 μl of PMSF solution (20 g/l acetonitrile) was added, and the mixture was incubated for 3 h at room temperature. The sulfonylated 32-mer peptide was flushed using pure nitrogen for 30 min and then treated with 20 μl of sodium hydroselenide (1 M) for 40 h at 37°C. The crude reaction mixture was first purified by centrifugation, dialyzed against sodium phosphate buffer (50 mM, pH 7.0) for 24 h, and finally lyophilized to obtain the selenopeptide.

The chelation of selenopeptide with copper was carried out in a $CuSO_4 \cdot 5H_2O$/aqueous solution (1:1.2, mol/mol). The pH was adjusted to 7.5 with NaOH (0.1 M), and incubated at 25°C for 8 h. Then, the mixed solution was concentrated at 55°C under vacuum. The concentrated solution was purified with a Sephadex G-50 column (1×90 cm) with 220-nm UV detection. Target peptide was collected and lyophilized and named as Se-Cu-32P.

The selenium content of the Se-Cu-32P was measured by hydride generation atomic fluorescence spectral analysis [16]. The copper content of the Se-Cu-32P was determined by elemental analysis. The fluorescence spectra of 32P and Se-Cu-32P were monitored in ultrapure water. All fluorescence measurements were carried out using an RF-5301PC spectrofluorometer. The samples were excited at 295 nm and the emission was recorded between 315 nm and 400 nm.

The GPX activities were determined by the method of Wilson *et al* [23]. The reaction was carried out at 37°C in 0.7 ml of solution containing 50 mM PBS (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 0.25 mM NADPH, 1 unit of glutathione reductase, and 10–50 μM Se-Cu-32P. The reaction was initiated by the addition of 0.5 mM H_2O_2. The activity was determined by the decrease of NADPH absorption at 340 nm. One unit of activity is defined as the amount of Se-Cu-32P that utilizes 1 μM of NADPH per minute. The activity was expressed in U/μM of Se-Cu-32P.

The assay of the kinetics of Se-Cu-32P was similar to that for native GPX [5]. The initial rates were measured by observing the increase of product absorption at 340 nm at several concentrations of one substrate while the concentration of the other substrate was kept constant. GSH concentrations were varied from 0.2 to 1.0 mM when H_2O_2 concentrations were fixed to 0.5, 1, and 2 mM; H_2O_2 concentrations were varied from 0.1 to 0.5 mM when GSH concentrations were fixed to 1, 2, and 3 mM. Kinetics data were analyzed by double-reciprocal plotting.

The SOD activity was determined by a standard indirect colorimetric assay that measures the level of inhibition of superoxide-induced reduction of colorless nitroblue tetrazolium dye to its oxidized blue formazan form [22]. One unit of SOD activity is defined as the amount of enzyme that inhibits the rate of nitroblue tetrazolium (NBT) reduction, under the specified conditions, by 50%. Generation of the superoxide anion was accomplished enzymatically by the reaction of xanthine and xanthine oxidase (XOD) [6].

Culture of Vero Cells and Evaluation of Antioxidant Effect

African green monkey kidney cell line vero cells were obtained from the Changchun Institute of Tumour. Iscove's Modified Dulbecco's Medium (IMDM), and fetal bovine serum were from Hyclone (Logan, UT). 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma. 15SeP and 17CuP were from our laboratory.

Vero cells were routinely cultured in IMDM with 10% fetal bovine serum. All cell cultures were performed at 37°C under a humidified atmosphere of 5% CO_2. Vero cell viability was assessed by the

Table 1. The amino acid sequences of 15P, 17P, and 32P.

Species	Peptide sequence
15P	WPFLRHNVYGRPRAS
17P	HGGHVHYIRSGAHVVYT
32P	HGGHVHYIRGGAHVVYTWPFLRHNVYGRPRAS

MTT method. Briefly, vero cells were seeded respectively at a density of 1.0×10^5 cells per well of a 96-well plate in 100-μl culture medium. After incubation for 24 h, vero cells were proincubated with Se-Cu-32P (15SeP or 17CuP) for 30 min. Then, cells were treated by the radical generation system XOD/Xanthine/Fe^{2+}. After cells were incubated for 24 h, they were washed three times with 100 μl of IMDM to avoid the disturbance of XOD/Xanthine/Fe^{2+}. Then, 20 μl of MTT solution (5 g/l) was added to each well, and the plate was incubated for 4 h at 37°C. The medium was discarded, and 100 μl of DMSO was added to dissolve the crystal. The absorption value at 570 nm was determined with a UV spectrometer.

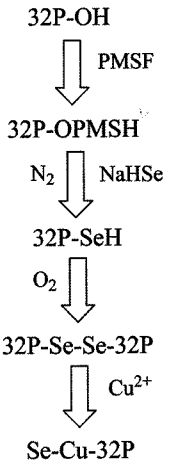
Results and Discussion

Synthesis and Characterization of Se-Cu-Peptide

The sequences of 15P, 17P, and 32P are listed in Table 1.

The 32P was synthesized by Fmoc chemistry in solid-phase synthesis. We used a BOP/HOBt/NMM activator in each case. Subsequently, peptides were cleaved from the resin, deprotected, and purified by HPLC. Finally, the collected solution was lyophilized, with typical yield of the peptide (43.6±0.3%). The molecular weight of 32P determined on a MALDI–TOF–MS spectrometer was 3,693.5±5 (Fig. 1), which is consistent with the calculated molecular weight of 32P (3,695.9).

The synthetic route of bifunctional Se-Cu-32P is shown in Scheme 1. The selenohydryl group was introduced into the peptide by selective sulfonylation of the serine hydroxyl of the 32P. The nucleophilic substitution by sodium hydroselenide gave 32P-SeH, which should be oxidized soon in air to form 32P-Se-Se-32P. The resultant compound, 32P-Se-Se-32P, contained a diselenium bridge (-Se-Se-) and



Scheme 1. Generation process of bifunctional Se-Cu-32P.

two fragments of 32P. To confirm this result, the selenium content was measured. The hydride generation atom fluorescence spectral analysis showed that the selenium content in selenopeptide is (2.2±0.1)%, corresponding to 2 selenium atoms per peptide molecule. This result demonstrated the existence of diselenium bridges, which is an oxide form of the peptide. In the native Cu,Zn-SOD, the Cu(II) is liganded by four histidines and makes up the active center of the protein [18]. In the present study, in order to mimic the active center of the native protein, we hoped for the copper content of the product to be not more than one Cu(II) per peptide molecule. Thus, 32P-Se-Se-32P was further chelated by a proper amount of Cu(II) to obtain the target peptide (Se-Cu-32P). The copper content of Se-Cu-32P was measured by elemental analysis. The result showed that the copper content in Se-Cu-32P is (0.69±0.01)%, corresponding to 0.8 Cu(II) per peptide molecule.

In order to demonstrate that Se and Cu ions were incorporated into the Se-Cu-32P, the fluorescence spectra of 32P and Se-Cu-32P were also determined by excitation of the samples at 295 nm. As shown in Fig. 2, the emission maximum of 32P and Se-Cu-32P appeared at 347 nm and

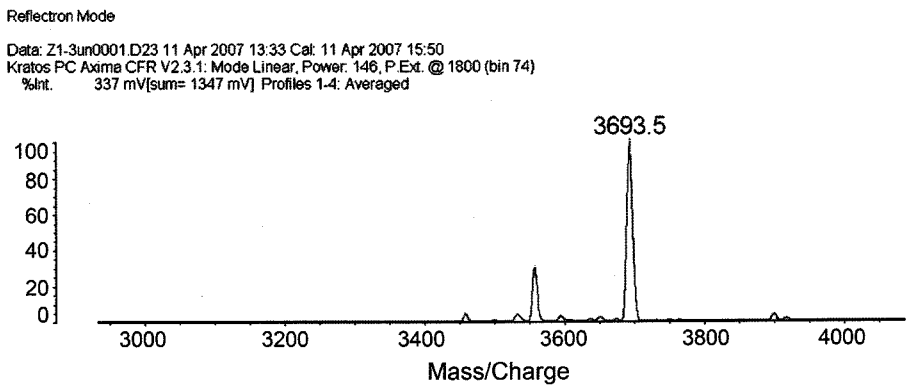


Fig. 1. Identification of 32P by MALDI–TOF–MS.

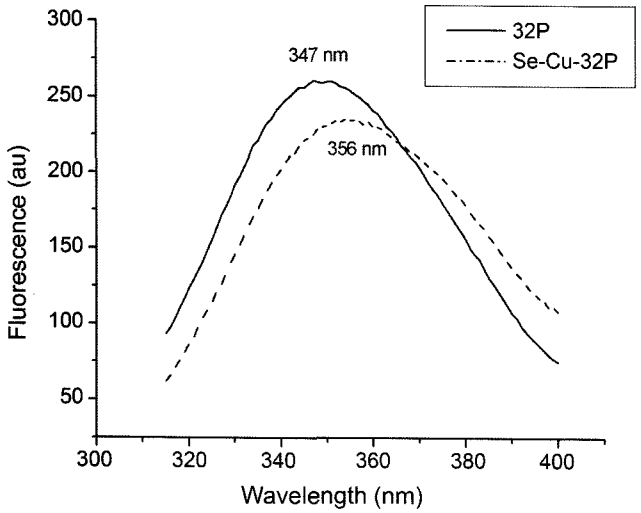


Fig. 2. Fluorescence emission spectra of 32P and Se-Cu-32P excited at 295 nm in aqueous solution.

356 nm, respectively. The red shift of the emission maximum indicated that the peptide conformation was changed as a result of incorporation of Se and Cu ions.

GPX-like Activity and Steady-State Kinetics of Se-Cu-32P

The GPX and SOD activities of Se-Cu-32P are listed in Table 2. The GPX activity of original 15SeP and the SOD activity of original 17CuP were included for comparison [11, 17]. The GPX activity of Se-Cu-32P for the reduction of H_2O_2 by GSH was found to be (146±0.9) U/μM, which was decreased by 44% compared with 15SeP. Simultaneously, the SOD activity of Se-Cu-32P was found to be (164±1.5) U/mM, which was decreased by 40% compared with 17CuP. To clarify this phenomenon that the catalytic activities of both GPX and SOD of this novel bifunctional mimic were decreased compared with their original peptides, we studied the steady-state kinetics of the mimic.

The steady state kinetics was observed for both H_2O_2 and GSH by varying one substrate concentration while another was fixed. Double-reciprocal plots of the initial velocity versus the concentration of substrates gave a family of parallel lines (Fig. 3), consistent with a ping-pong mechanism of the native GPX and the GPX mimic, 15SeP. Based on Eq. (1), the kinetic parameters were

Table 2. The GPX and SOD activities of 15SeP, 17CuP, and Se-Cu-32P.

Species	GPX activity (units/μM)	SOD activity (units/mM)
15SeP	260 [16]	ND
17CuP	ND	272±2.2
Se-Cu-32P	146±0.9	164±1.5

All data are presented as means±SD.

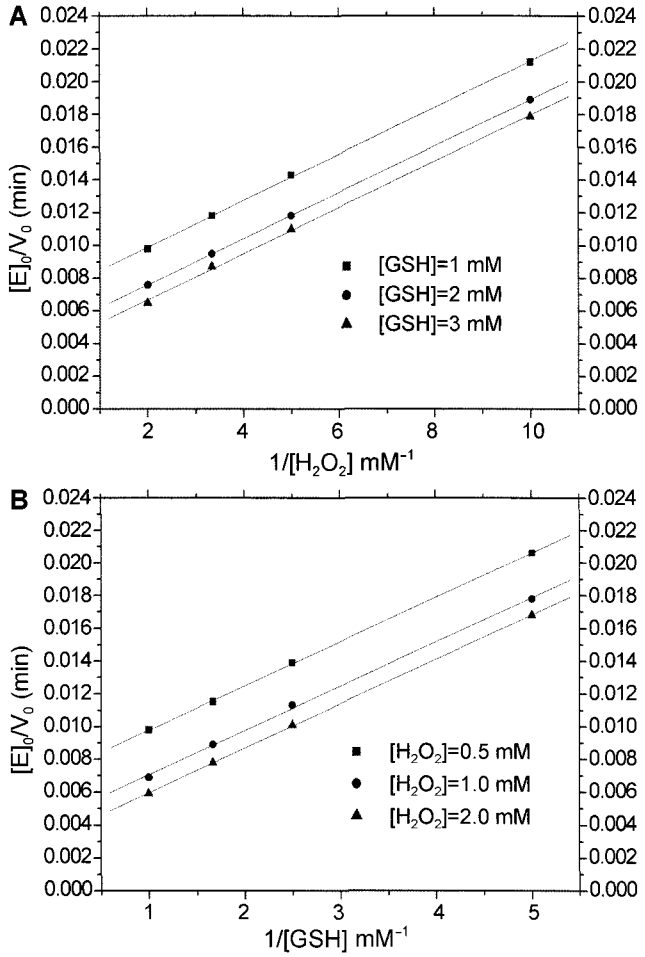


Fig. 3. Double-reciprocal plots for the reduction of H_2O_2 by GSH catalyzed by Se-Cu-32P.
A. $[E]_0/V_0$ versus $1/[H_2O_2]$ (mM^{-1}) at [GSH]=1 (■), 2 (●), and 3 mM (▲);
B. $[E]_0/V_0$ versus 1/[GSH] (mM^{-1}) at $[H_2O_2]$=0.5 (■), 1 (●), and 2 mM (▲).

calculated from the double-reciprocal plots. In this formula, v_0 is the initial reaction velocity, $[E]_0$ is the initial enzyme mimic concentration. The pseudo first-order rate constant k_{max} and the apparent Michaelis constants K_{GSH} and $K_{H_2O_2}$ were found to be 556 min^{-1}, 2.04×10^{-3} M, and 1.36×10^{-3} M, respectively. The apparent second-order rate constants k_{max}/K_{GSH} and $k_{max}/K_{H_2O_2}$ were found to be 2.43×10^5 $M^{-1}min^{-1}$ and 4.09×10^5 $M^{-1}min^{-1}$, respectively. Upon comparing the apparent second-order rate constants k_{max}/K_{GSH} and $k_{max}/K_{H_2O_2}$ of native GPX and 15SeP with those of Se-Cu-32P (Table 3), we found that both apparent second-order rate constants were obviously decreased. This result indicates that the binding ability of Se-Cu-32P with GSH and H_2O_2 was weakened and this induced the decrease of the GPX activity directly. When the 15SeP and 17CuP were conjoined covalently, their original conformations could be changed. So the GPX and SOD activities of Se-Cu-32P were somewhat decreased compared with their original peptides.

Table 3. Kinetic parameters of the Se-Cu-32P.

Species	k_{max} (min^{-1})	K_{GSH} (M)	k_{max}/K_{GSH} ($M^{-1}min^{-1}$)	$K_{H_2O_2}$ (M)	$k_{max}/K_{H_2O_2}$ ($M^{-1}min^{-1}$)
Se-Cu-32P	5.56E+02	2.04E-03	2.43E+05	1.36E-03	4.09E+05
15SeP	2.33E+03	1.87E-02	1.28E+08	9.64E-03	2.42E+08
Native GPX			E+08		E+09

$$v_0/[E]_0=\frac{k_{max}[GSH][H_2O_2]}{K_{H_2O_2}[GSH]+K_{GSH}[H_2O_2]+[GSH][H_2O_2]} \quad (1)$$

Protection of Vero Cells Against ROS Damage by Se-Cu-Peptide

The biological effects of Se-Cu-32P, 15SeP, and 17CuP were evaluated simultaneously by the protection of vero cells against ROS damage. As shown in Fig. 4, vero cells were damaged distinctly by XOD/xanthine/Fe^{2+}, and the cell viability was decreased by nearly 45%. The Se-Cu-32P group and the 15SeP group showed obvious protective effects and the changes of cell viability percentage were shown to be dose-dependent. Morever, the protective effect of the Se-Cu-32P group was better than that of the 15SeP group in identical concentrations. However, the 17CuP group not only had no protective effect but also showed injury effect because of the accumulation of H_2O_2. In the XOD/xanthine/Fe^{2+} damage system, the XOD-catalyzed reaction is known to initially produce superoxide anions, which are spontaneously reduced to H_2O_2 by SOD, and then H_2O_2 is converted to OH· in the presence of Fe^{2+}. Consequently, there exists simultaneously $O_2\cdot^-$, H_2O_2, and OH· in the system. Se-Cu-32P could eliminate not only O^{2-} but also H_2O_2 owing to both its SOD and GPX activities. 15SeP could clear H_2O_2, but it could not dismute $O_2\cdot^-$. 17CuP could convert $O_2\cdot^-$ to H_2O_2, but the H_2O_2 could not be eliminated immediately. Therefore, the bifunctional enzyme Se-Cu-32P had better protective effect than 15SeP and 17CuP in the XOD/xanthine/Fe^{2+} damage system. Under the condition of the same concentration, although the GPX activity of the Se-Cu-32P was lower than that of the15SeP, the Se-Cu-32P had better protective ability than the 15SeP. This illustrated the importance of the synergism of the GPX activity and the SOD activity. Thus, the novel bifunctional enzyme Se-Cu-32P is better than single functional enzymes in the elimination of ROS.

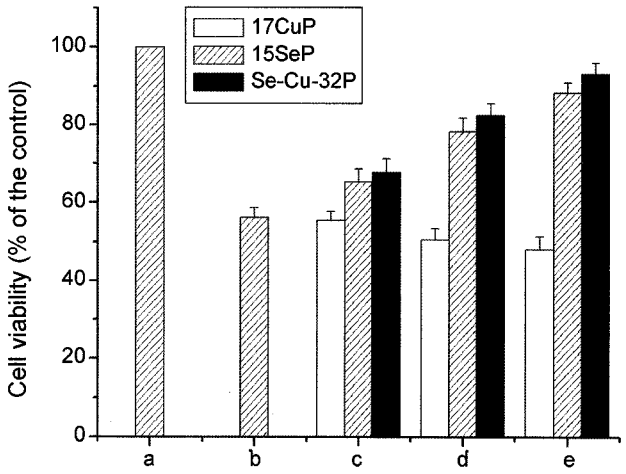


Fig. 4. Effects of Se-Cu-32P, 15SeP, and 17CuP on the viability of vero cells induced by XOD/xanthine/Fe^{2+} injury.
The cells were treated as indicated in Materials and Methods. (a) Control; (b) XOD/xanthine/Fe^{2+} injury; (c) XOD/xanthine/Fe^{2+}+50 nM Se-Cu-32P (15SeP or 17CuP); (d) XOD/xanthine/Fe^{2+}+100 nM Se-Cu-32P (15SeP or 17CuP); (e) XOD/xanthine/Fe^{2+}+200 nM Se-Cu-32P (15SeP or 17CuP). Percentage of viable cells was counted by MTT assays. Data are expressed as mean±SD for 3 independent preparations.

A novel bifunctional enzyme mimic, Se-Cu-32P, was successfully generated and displayed both GPX and SOD activities. Moreover, the novel mimic was demonstrated to be able to better protect vero cells from the injury induced by the XOD/xanthine/Fe^{2+} damage system than its parents, indicating that the novel peptide has potential in medicinal application.

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