Production of Selenium Peptide by Autolysis of *Saccharomyces cerevisiae*

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Abstract Selenium-containing peptide (selenium peptide) was produced by autolysis of total proteins of *Saccharomyces cerevisiae* grown with inorganic selenium. Selenium peptide exhibited antioxidant activity as a glutathione peroxidase (GPx) mimic, and its activity was dependent on the hydrolysis methods. The GPx-like activity of the hydrolyzed selenium peptide increased 2.7-folds when digested by protease, but decreased by acid hydrolysis. During the autolysis of the yeast cell, the GPx-like activity and selenium content increased 4.3- and 2.3-folds, respectively, whereas the average molecular weight (MW) of selenium peptide decreased 70%. The GPx-like activity was dependent on the MW of selenium peptide and was the highest (220 U/mg protein) at 9,500 dalton. The maximum GPx-like activity (28,600 U/g cell) was obtained by 48 h of autolysis of the cells, which were precultured with 20 ppm of selenate. Selenium peptide showed little toxicity, compared with highly toxic inorganic selenium. These results show the potential of selenium peptide as a non-toxic antioxidant that can be produced by simple autolysis of yeast cells.

Key words: Selenium, glutathione peroxidase. GPx mimic, autolysis, antioxidant

Selenium is a trace element and has various functions, including antioxidant activity, cancer prevention, suppression of cancer spreading, and improvement of immune system [8, 15]. Deficiency of selenium in the human body causes serious diseases such as heart (Kesan) and joint (kashin-Beck) diseases [26].

Various proteins containing selenium have antioxidant properties and include glutathione peroxidase (GPx),

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thioredoxin reductase, selenoprotein P, selenoprotein W, and selenoprotein R [15]. In the case of GPx, selenium exists as a selenocysteine in the active site of the GPx. Selenocysteine reduces the oxidized products by oxidizing two gluthathiones [15]. With this antioxidant characteristic, GPx removes hydrogen peroxide, lipid peroxide, and phospholipid peroxide, and protects DNA, lipid, and protein from oxidative free radicals [1, 6, 27].

However, practical use of GPx as an antioxidant is limited, since GPx is an unstable and large protein with molecular weight of 84 kD. Recently, there have been great increases in the development of GPx mimics [10, 14, 18, 24, 25]. Most work of GPx mimics have been focused on the chemical synthesis of organic selenium and analysis of GPx-like activity of GPx mimics. Another approach is to biologically produce organic selenium.

In yeast, inorganic selenium is converted into selenomethionine and incorporated in protein. Indeed, yeast is a very effective organic selenium producer, since yeast can be produced easily in large scale [13], and more than 80% of the inorganic selenium is converted into selenomethionine and incorporated in proteins in the yeast [23]. Since the selenium-containing proteins or selenium proteins are least toxic to cells compared with selenomethionine or inorganic selenium, it would be of great interest if the selenium protein possesses antioxidant property [21]. Furthermore, small fragments of selenium-protein, so-called selenium peptide, would be a more practical and suitable form of ingredient than yeast cells if it would be used in foods or in cosmetics.

Very few works have been reported on the production and characterization of selenium peptide from the yeast. In this work, various sizes of selenium-containing peptide (selenium-peptide) were produced by various hydrolysis methods of cultured yeast and the GPx-like activity of the selenium peptide was investigated.

MATERIALS AND METHODS

Cultivation and Separation of Selenium Protein

Saccharomyces cerevisiae (ATCC 7752) was cultured in 50 ml YM broth containing 50 ppm of sodium selenate (250-ml flask) at 250 rpm and 30°C for 48 h. After harvesting the cells by centrifugation at 3,000 ×g for 30 min, cell pellets were washed twice by distilled water, followed by sonication for cell disruption (Sonifier 450, Branson, U.S.A.). Cell debris was removed by centrifugation (3,000 ×g for 30 min) and proteins were precipitated by ammonium sulfate (80%, 30 min). After dialysis, proteins were freeze-dried. All the chemicals and enzymes were purchased from Sigma.

Hydrolysis of Selenium Protein

Selenium protein was hydrolyzed at various concentrations of HCl at 80°C for 1 h, followed by neutralization with 5 N NaOH. For enzymatic hydrolysis, selenium protein was hydrolyzed by protease K or pronase E at 37°C for 3 h, followed by inactivation of the protease by heating at 90°C for 10 min. For autolysis, cultured yeast cells were harvested. When necessary, cells were sonicated under aseptic conditions. Cell density was maintained at 80–100 g/l for autolysis. After the addition of various concentrations of NaCl and ethanol, cells were incubated for 48 h at 50°C and 250 rpm, followed by removing cell debris by centrifugation (3,000 ×g, 30 min).

Separation of Selenium Peptide

The fractions of hydrolyzed selenium protein were obtained by using size exclusion chromatography. All chromatographic steps were performed using a fast protein liquid chromatography (FPLC) equipped with data view software (BioLogic LP, Bio-Rad, U.S.A.). Sample was loaded to a Superdex 30 column (1.6×60 cm) (Amersham Biosciences, U.S.A.) and ionized water at a flow-rate of 0.6 ml/min was used to elute the column with detection at UV 280 nm. Fractions of 6 ml were collected and freeze-dried.

Analysis

Antioxidant activity of selenium peptide was measured by glutathione peroxidase activity according to the methods of Paglia and Valentine [16]. Sample (0.05 ml) was added to 0.4 ml of potassium phosphate buffer solutions (50 mM, pH 7) containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 mM glutathione, and 1 U/ml glutathione reductase. After 10 min of incubation at 37°C, 0.05 ml of H_2O_2 (5 mM) was added, and the decrease in optical density (340 nm) for 5 min was measured. One unit was defined as 1 µmol of NADPH consumed per min. Protein was analyzed by BCA (bicinchoninic acid) assay kit (Pierce, U.S.A.).

For the determination of selenium in the cell, cells were digested with 60% HNO_3 for 12 h at 60°C. Selenium protein

was diluted in 1% HNO_3 for measurement. Graphite furnace atomic absorption spectrophotometerg (AAS 5EA, Analytical Jena, Germany) was used for selenium quantification. Palladium (10 ppm) was added to the sample for stabilization [4, 9].

The molecular weight of the selenium peptide was analyzed by HPLC with a Waters Ultrahydrogel 250 gel permeation chromatography (GPC) column (1 ml/min; 10 mM potassium phosphate buffer, pH 7) at UV 280 nm. Average molecular weight (MW) was calculated as $\Sigma(MW_i \times concentration_i)/\Sigma concentration_i$, where MW_i and $concentration_i$ indicate the MW and concentration of each peak, respectively, in the GPC diagram.

RESULTS AND DISCUSSIONS

### Acid and Enzymatic Hydrolysis of Total Proteins

The effectiveness of various methods of hydrolysis was investigated, including digestion by HCl, protease, and autolysis. By HCl hydrolysis, the GPx-like activity of selenium peptide decreased as the concentration of HCl increased (Fig. 1). This decrease was presumably due to degradation of amino acids such as methionine or cysteine, which contains a thiol group. Since selenomethionine contains selenium in place of sulfur, the degradation of selenomethionine by HCl hydrolysis probably resulted in the loss of the GPx-like activity.

Hydrolysis of total protein by protease significantly increased the GPx-like activity of selenium peptide (Fig. 2). Almost 2.7-folds increase in the GPx-like activity was observed by hydrolyzing with either pronase E or protease K. Enzymatic hydrolysis decreased the size of the selenium peptide, indicating that the GPx-like activity of selenium
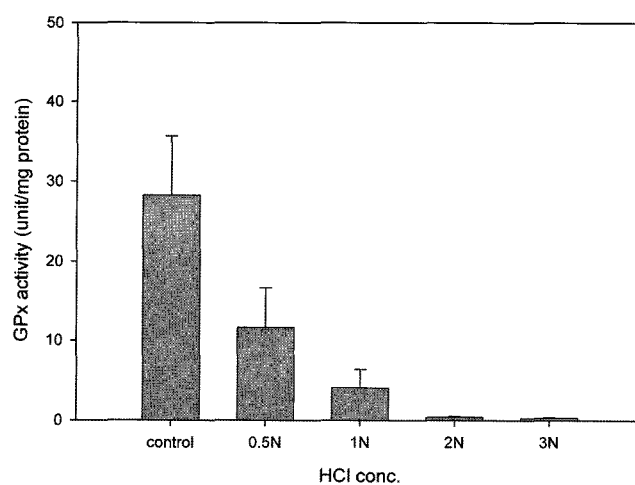
Fig. 1. Effect of HCl concentration on the GPx-like activity of selenium peptide prepared by HCl hydrolysis.

Total protein was hydrolyzed for 1 h at various concentrations of HCl (Control; Total protein without HCl hydrolysis).
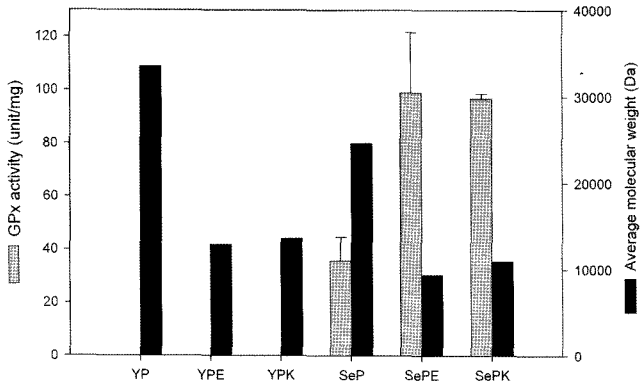
Fig. 2. GPx-like activity of selenium peptide prepared by various proteases.

Proteins were prepared from yeast cultured without (YP, YPE, YPK) and with 50 ppm sodium selenate (SeP, SePE, SePK). YP, yeast total protein; YPE, yeast protein hydrolysate by pronase E; YPK, yeast protein hydrolysate by protease K; SeP, total selenium protein; SePE, Selenium protein hydrolysate by pronase; SePK, Selenium protein hydrolysate by protease K.

peptide was dependent on the size of the peptide. A similar observation of increased activity of bioactive peptide by enzymatic hydrolysis has previously been reported [3, 11].

Autolysis of Yeast Cells

Although the enzymatic digestion of the total protein resulted in the higher GPx-like activity, the addition of enzymes could increase the production cost of selenium peptide. When yeast cells are starved of nutrients under appropriate temperature, high molecular compounds such as protein, polysaccharide, and nucleic acids are hydrolyzed. This so-called autolysis process can provide advantages since no exogenous enzymes are necessary.

In order to increase the effectiveness of the autolysis, mechnical sonication was also attempted. As shown in Fig. 3, sonication increased GPx-like activity 2-folds. In addition, more proteins were released by sonication. Similar
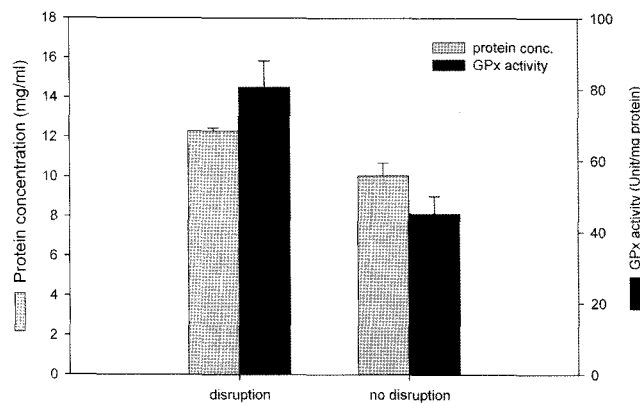
Fig. 3. Effect of cell sonication on the GPx-like activity.
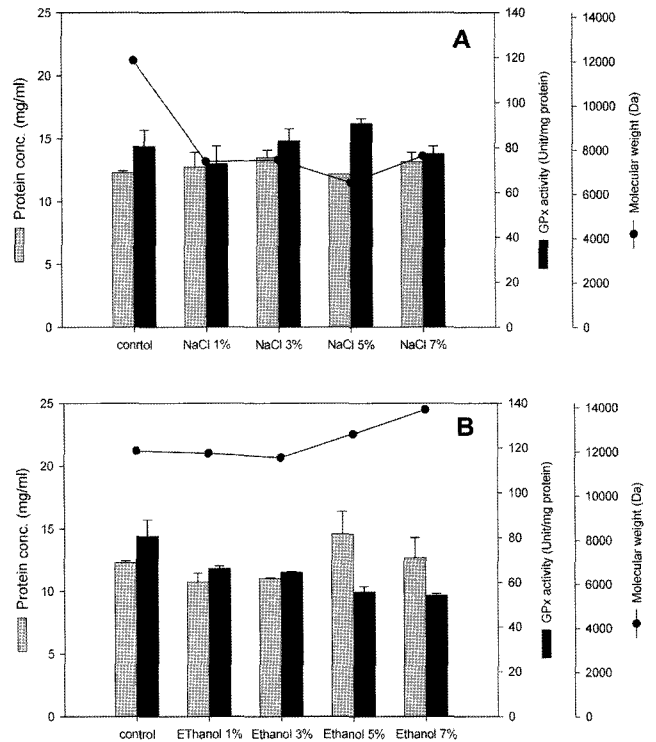
Fig. 4. Effect of NaCl (**A**) and ethanol (**B**) during autolysis on the GPx-like activity.

effects of mechanical treatment on the acceleration of autolysis have been reported [5] and mechanical disruption of cell wall is known to accelerate the activation of proteases [2].

During the autolysis, addition of accelerators has been reported to increase autolysis and to prevent contamination [2, 22]. To increase the yeast autolysis and GPx-like activity, various concentrations of NaCl or ethanol were added. As shown in Fig. 4, the addition of NaCl decreased the average molecular weight significantly. At 5% NaCl, 40% decrease in molecular weight and 20% increase of GPx-like activity, respectively, were observed. However, high concentration of ethanol increased the MW of the selenium peptide and decreased the GPx-like activity, indicating inhibition of autolysis at high ethanol concentration. When mixtures of ethanol and NaCl were added, no increase of GPx-like activity was observed (data not shown).

Effects of Selenium on Selenium Peptide Production

The concentration of selenium in the culture medium significantly affected the selenium contents of the selenium peptide as well as the cell growth. As shown in Table 1, growth of cells was significantly inhibited even at 10 ppm of sodium selenate, indicating the toxicity of inorganic selenium. At 20 ppm, selenium content in the protein was the highest and the GPx-like activity reached its maximum at 20 ppm. Accordingly, based on the total GPx-like activity,

Table 1. Effect of initial selenium concentration (sodium selenate) on the GPx-like activity and selenium content of peptide obtained by autolysis.

	Selenium concentration	0 ppm	10 ppm	20 ppm	50 ppm	100 ppm
Yeast culture	Growth (OD 600 nm (g/l))	11.5 (5.9)	6.5 (3.3)	5.5 (2.8)	5.2 (2.8)	4.4 (2.2)
	Se in cells (μg Se/g cells)	1.6 ±23.6	1491.8 ±54.6	1138.1 ±47.0	706.7 ±59.7	1037.78±94.5
After autolysis	Total protein (mg protein/mg cells)	63.2	63.4	60.5	60.9	58.8
	Se in protein (μg Se/mg protein)	0.2 ±0.02	1.2 ±0.07	8.2 ±0.4	6.1 ±0.2	4.1 ±0.2
	Se in cells (μg Se/g cells)	9.6	71.4	498.0	373.2	243.7
	Total Se (μg/l)	55.9	237.1	1382.6	1030.4	544.9
	GPx activity (unit/mg protein)	ND	27.1 ±2.6	182.1 ±4.3	92.1 ±5.7	115.1 ±2.6
	GPx activity in cells (unit/g cells)	ND	1664.5	11052.3	5611.3	6768.6
	Total GPx activity (unit/l)	ND	5528.2	30683.8	15492.4	15136.3

the optimum sodium selenate concentration was found to be 20 ppm. Sodium selenate and sodium selenite are inorganic seleniums that have been widely used for the production of selenium yeast. When incubated with yeast, sodium selenite binds to protein as organic form; however, it also binds to cell wall as inorganic form [7]. On the other hand, sodium selenate binds to cellular protein as organic form, and therefore, sodium selenate is preferred for biological uses such as the production of organic selenium from yeast. At 20 ppm of sodium selenate, the content of selenium in protein was the highest, and the GPx-like activity was superior (Table 1).

Change of Selenium Peptide Property During Autolysis
To investigate the time-course behavior, yeast was cultured at 20 ppm of selenium, followed by homogenization and autolysis. In 6 h of initial period, the GPx-like activity and the selenium content in peptides increased, whereas rapid reduction of average molecular weight was observed (Fig. 5). Although no significant difference in the protein release was observed by autolysis, the selenium content and GPx-like activity increased approximately 2.2- and

Fig. 5. Time-course behavior of selenium peptide production by autolysis of *S. cerevisiae.*
Yeast was cultured with 20 ppm sodium selenate and autolysis started at time 0.

2.3-folds, respectively (Table 2). The increase by such autolysis appeared to be closely associated with the release of organic selenium compounds incorporated into cell wall and also with the reduction of molecular weight of selenium peptide during autolysis.

Selenium contained in selenium yeast is mostly selenomethionine, and the fraction of selenium compounds present in water is 10–15% of the total protein. It has also been reported that the fraction of selenium incorporated into cell wall is more than 60% of the total selenium, most of them being selenomethionine [17]. This shows the possibility that the enzymatic hydrolysis of cell wall and total protein during autolysis could facilitate the production of low molecular weight organic selenium.

Toxicity of Selenium Peptide
To evaluate the toxicity of selenium compounds, various selenium compounds (10 ppm) were added to yeast, and they were incubated and their growth was examined. Sodium selenite inhibited 87.2% of the growth, indicating the toxicity of sodium selenite. Even the selenomethionine decreased the cell growth rate (14.9%). However, no significant inhibition (4.7%) was observed with selenium peptide (Fig. 6). When selenium peptide was added to yeast, growth inhibition was observed during the initial growth period (Fig. 6). However, similar inhibition was detected, when a peptide (not selenium peptide) was added; therefore, this could be due to a high concentration of protein (approximately 2,600 ppm). Low toxicity is a requirement of an additive in foods and in health care [20]. In that sense, selenium peptide would be an excellent additive as a selenium supplier.

Properties of Selenium Peptide of Various Molecular Weights
The GPx-like activity and selenium content of selenium peptide were investigated. By using FPLC, 8 selenium peptides were fractionated (Fig. 7A). Each fraction was analyzed for its molecular weight by size exclusion chromatography and GPx-like activity. As shown in Fig. 7B, the highest
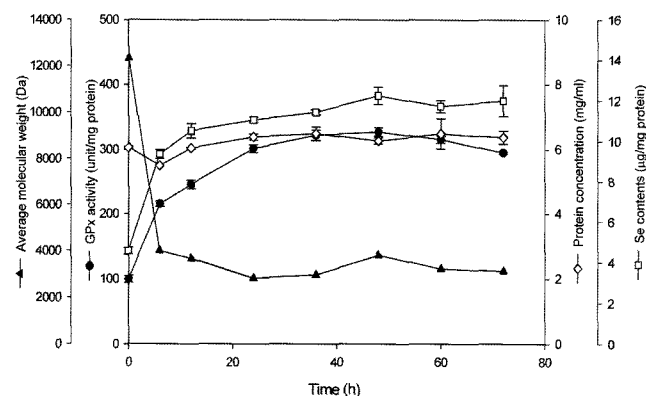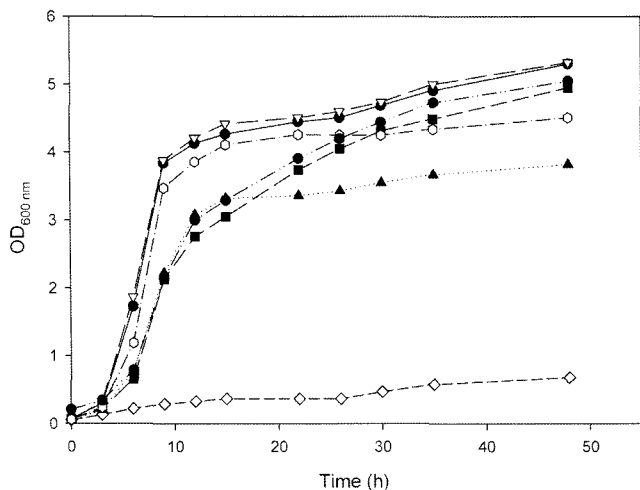
Table 2. Yield of selenium peptide obtained by autolysis after culturing at 20 ppm of selenium selenate.

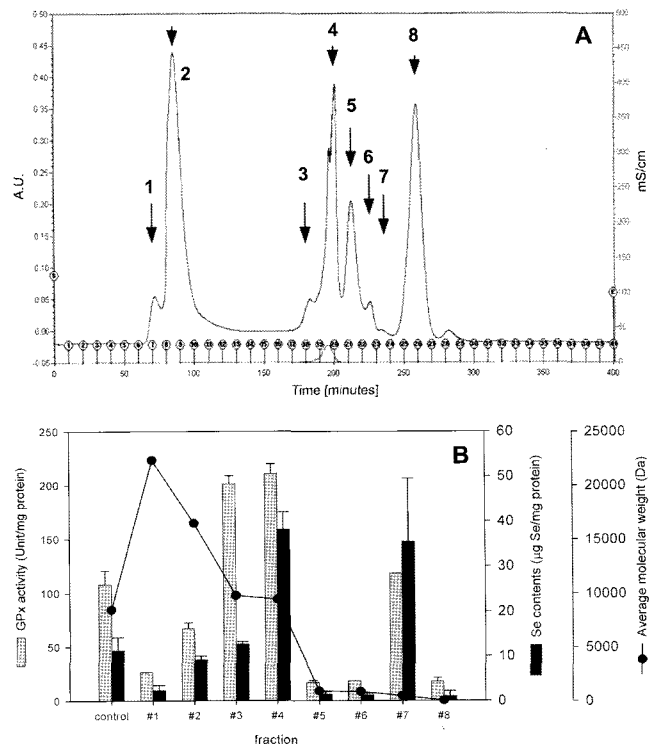|	Before autolysis	After autolysis (48 h)	Ratio (before:after)
Released protein (mg protein/g cell)	84.8±0.5	87.7±1.3	1:1.0
Se conc. (mg Se/mg protein)	6.4±0.2	14.2±0.1	1:2.2
Total Se recovery (mg Se recovery/g cell)	538.3	1,241.0	1:2.3
GPx activity (Unit/mg protein)	99.3±5.3	327.0±6.5	1:3.3
Total GPx activity (Unit/g cell)	8,421.4	28,687.0	1:3.4

GPx activity was observed in the 3rd and 4th peaks. The GPx-like activity increased as the molecular weight of selenium peptide decreased, except the 7th peak. The 7th peak appears to be a very short peptide or selenomethionine. With this peak fraction, despite of the high selenium content, the GPx-like activity was lower than that the 3rd and 4th peak fractions, indicating that a certain length of peptide was essential for high GPx-like activity. The average GPx-like activity and selenium content of total protein hydrolysate were 120 (U/mg protein) and 12 (μg selenium/mg protein), respectively. The average activity of total hydrolysate was lower than that of the maximum activity obtained after 48 h of autolysis (Table 2 and Fig. 5). This decrease might be due to the loss of the small organic selenium fraction, presumably selenomethionine and/or selenocysteine, by the filtration and precipitation employed in the sample treatment for FPLC and GPC analyses. Since the 3rd and 4th peaks (approximately 9,500 dalton) showed 2-folds higher GPx-like activity than the average total lysate, optimization of the autolysis conditions to increase the 3rd and 4th fractions could enhance the total yield of the GPx-like activity of selenium peptide. Similar to the GPx-like activity of the selenium peptide from yeast, the peptide

obtained from soy sauce or rice wine showed antitumor and antioxidant properties [12, 19].

In this study, selenium peptide was produced as a GPx mimic by culturing yeast with inorganic selenium, followed by autolysis. The autolysis process was analyzed and optimized, based on the protein yield and GPx-like activity. A simple autolysis step could produce selenium peptide with high GPx-like activity. Selenium peptide produced by this method showed no toxicity compared with highly toxic inorganic selenium. Considering the importance of selenium in diet, selenium peptide could be an excellent dietary form of selenium. Moreover, a high GPx-like activity shows the potentials of selenium peptide as a novel antioxidant in various fields, including foods and cosmetics.

Fig. 7. The properties of selenium peptide, separated using FPLC (BioLogic LP system, Superdex 30, 1.5×60 cm).
A. Eight peaks were observed by FPLC separation. B. Numbers in the X-axis correspond to the peak in the (A) plot. The molecular weight of each peak was determined by gel permeation chromatography. Control means the hydrolysate before FPLC fraction.

Fig. 6. Effect of various selenium species on the growth of S. cerevisiae.
—●— control (no addition of selenium), - -▽- — methionine, - -■- — peptide, - - -◇- - sodium selenate, ▲ sodium selenite, -○- - selenomethionine, - -●- — selenium peptide.

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