BioMetals 1997, **10**, 11–22

Effects of selenium and serum on selenoprotein W in cultured L8 muscle cells*

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Received 2 January 1996; accepted for publication 11 June 1996

When rat L8 muscle cells were cultured to examine the effects of serum and selenium concentration on selenoprotein W levels and glutathione peroxidase (GPX) activities, no significant differences ($P > 0.05$) were found in selenoprotein W levels and GPX activities during differentiation. With three different forms of selenium, selenoprotein W levels and GPX activities were shown to increase in L8 myotubes cultured in media with these selenocompounds. Selenite was utilized more efficiently than selenocysteine for both selenoprotein W and GPX activity, but selenium as selenomethionine was less available. Both the protein content and mRNA levels for selenoprotein W were affected by the selenium content of the media. Northern blot data indicated that the expression of selenoprotein W mRNA increased significantly when L8 myotubes were cultured with selenium ($P > 0.05$). L8 myotubes cultured in 10% calf serum (CS) versus 2% CS with or without addition of 10^{-8} M selenium indicated that the increase of selenoprotein W level in L8 myotubes cultured with higher serum concentration (10% CS) is due to the higher selenium concentration in media rather than serum itself.

Keywords: glutathione peroxidase, myotubes, Northern blots, selenite, selenocysteine, selenomethionine, selenoprotein W, Western blots

Introduction

Several selenoproteins have been found in mammalian tissues, but only two groups of these have known functions. There is a family of selenium-dependent glutathione peroxidases and to date four members have been identified. The cellular glutathione peroxidase (cGPX) was the first one of this family discovered (Rotruck *et al*. 1973). Subsequently, the plasma GPX (pGPX, Takahashi *et al*. 1987), phospholipid hydroperoxide GPX (PHGPX, Ursini *et al*. 1985) and gastrointestinal tract specific GPX (GPX-GI, Chu *et al*. 1993) have been identified. The other known mammalian selenoenzymes are types I (Arthur *et al*. 1990), II (Davey *et al*. 1995) and III (Croteau *et al*. 1995) iodothyronine deiodinases. Types I and II deiodinases convert thyroxine to triiodothyronine and type III converts thyroxine to the inactive metabolite called reverse T_3.

There are two mammalian selenoproteins identified in which there is no known function. The most studied one is selenoprotein P, which contains more selenium than any known protein. The cDNA for selenoprotein P has been cloned and sequenced (Hill *et al*. 1991) and contains 10 TGAs in the open reading frame. Thus it contains 10 selenocysteine residues in its primary structure. A second selenoprotein, called selenoprotein W, was recently characterized from our laboratory (Vendeland *et al*. 1993); this contains only 87 amino acid residues (Vendeland *et al*. 1995). Evidence has been presented for a number of other selenium-containing

*Published with the approval of Oregon State University Experiment Station as paper number 10, 850. This research was supported by Public Health Service Research Grant number DK 38306 from the National Institutes of Diabetes and Digestive and Kidney Diseases.

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proteins, but none of these has been fully characterized (Behne *et al*. 1988; Kalcklosch *et al*. 1995).

Cell cultures have been extremely useful in studying selenium metabolism; a few examples are now given. Cell cultures were used to demonstrate that selenite was more effective than selenomethionine (Semet) for increasing GPX activity (White and Hoekstra 1979; Beilstein and Whanger 1987). The conversion of Semet to selenocysteine was shown to be influenced by the methionine content of the medium (Beilstein and Whanger 1987). Various selenium compounds have been studied in transsulfuration defective cells (Beilstein and Whanger 1992), and again higher levels of Semet than either selenite or selenocysteine were required to induce the same amount of GPX activity. Kidney cells in culture were used to study selenium regulation of mRNAs of GPX and 5′ deiodinase (5′-DI, Gross *et al*. 1995). Recognition of UGA as a selenocysteine codon in type I iodothyronine deiodinase was demonstrated *in vitro* (Berry *et al*. 1991), further demonstrating the value of this technique. Hence, cell cultures were used in the present investigation to study the influences of selenium and serum on selenoprotein W levels, with the aim of understanding factors which may regulate selenoprotein W concentrations.

The L8 muscle cell line was originally established in 1969 by serial passage of myoblasts isolated from primary rat skeletal muscle cultures prepared from newborn Wistar rats (Yaffe 1968). Of the tissues examined, selenoprotein W was highest in muscle from both rats (Yeh *et al*. 1995) and sheep (Yeh *et al*. 1996). Rat muscle appears to be the most desirable tissue for studying the influences of selenium status and serum concentration on selenoprotein W levels; thus, rat L8 skeletal muscle cells were used in the present investigations.

Materials and methods

Materials

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Calf serum (CS) was purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin solution and trypsin were purchased from GIBCO (Grand Island, NY). Rainbow molecular weight marker and the ECL Western blotting detection system were purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and bovine serum albumin (BSA) were purchased from Bio-Rad (Richmond, CA). Cell culture petri dishes were purchased from Corning (Corning, NY). Nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH). GeneScreenPlus nylon membrane was purchased from DuPont/NEN (Boston, MA).

Cell culture

Undifferentiated L8 myoblasts were cultured in basal medium (DMEM, 100 units penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 44 mM sodium bicarbonate, pH 7.4) supplemented with 10% CS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed every two days until cells reached confluence, and was then replaced with differentiation medium (basal medium with 2% CS) to induce differentiation. Microscopic examination was used to monitor cell differentiation.

Cell treatments

Experiment 1. This experiment was conducted to evaluate the influences of differentiation and selenium on selenoprotein W levels. Basal medium supplemented with 10% CS was used to permit myoblast proliferation, after which the medium was replaced with DMEM supplemented with 2% CS (differentiation medium) to induce differentiation. Cells were collected every 2 days during differentiation to examine the effect of differentiation on selenoprotein W. In addition, cells were cultured in basal medium supplemented with 10% CS to reach confluence, then myoblasts were cultured in differentiation medium with various selenium concentrations (10^{-6} M, 10^{-7} M or 10^{-8} M Se as selenite) for 6 days to examine the long-term effects of selenium on selenoprotein W.

Experiment 2. Effects of different forms of selenium on selenoprotein W levels and GPX activities were examined. Four-day differentiated myotubes were incubated in differentiation medium supplemented with various concentrations (10^{-6} M, 10^{-7} M or 10^{-8} M) of selenium as selenite, L-selenomethionine or L-selenocystine for 0, 16, 24 and 48 h. L8 myotubes were also incubated in differentiation medium supplemented with 10^{-6} M sulfur as L-cysteine and L-methionine.

Experiment 3. The effect of serum on selenoprotein W levels and GPX activities was investigated. Four-day differentiated myotubes were incubated with either basal medium (without serum) or differentiation medium. Each type of medium was supplemented with various concentrations of selenium (10^{-6} M, 10^{-7} M and 10^{-8} M) as selenite for 0, 16, 24 and 48 h.

After treatments, each plate of cells was washed with ice-cold 1X PBS (pH 7.4) three times; homogenate buffer (20 mM Tris [pH 7.5], 0.25 M sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSF, 50 mM β-mercaptoethanol and 25 μg ml^{-1} eupeptin) was then added to each plate and cells were scraped into tubes. After sonication for 10 sec, the mixtures were centrifuged at 17 000 × *g* for 10 min at 4°C. Protein content was measured in the supernatants by the

dye-binding assay of Bradford (1976) using bovine serum albumin (Bio-Rad, Richmond, CA) as a standard. Cell supernatants were then used to measure selenoprotein W level by Western blotting technique and GPX activities by a coupled enzyme procedure. Selenium content was determined in the media before use to ensure the correct selenium concentration.

Western blot assay

This assay was conducted as described previously (Yeh *et al*. 1995). Briefly, the cell supernatants (150 μg protein each) were electrophoretically separated on SDS–polyacrylamide 7.5 to 15% gradient gels as described by Laemmli (1970). Proteins were transferred onto a nitrocellulose membrane (0.2 μm; BA-S83, Schleicher & Schuell, Keene, NH) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 4°C according to method of Towbin *et al*. (1979). After transfer, membranes were incubated with blocking solution (5% non-fat dried milk in TTBS [0.05% Tween 20 in TBS; pH 7.5]) at 25°C for 1 h, then incubated with rabbit anti-selenoprotein W polyclonal antibody at 25°C for 1.5 h. Following three washes, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Bio-Rad, Richmond, CA). Membranes were then washed four times with TTBS and specific bonding of anti-selenoprotein W antibody onto the membrane was detected by the ECL detection system (Amersham, Arlington, IL). The relative amounts of selenoprotein W present in the various samples were estimated by densitometric scanning of the X-ray film using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuanNT program (Molecular Dynamics, Sunnyvale, CA).

Glutathione peroxidase activity and selenium content

GPX activity was measured by an enzyme coupled method with glutathione reductase, utilizing hydroperoxide as substrate (Paglia and Valentine 1967) and a DU Series 60 Spectrophotometer (Beckman Instruments, Fullerton, CA). After digestion of media with nitric and perchloric acids, the selenium content was determined fluorometrically by a semi-automated method (Brown and Watkinson 1977) using an Alpchem II system (Alpchem. Corp., Milwaukee, OR).

Total RNA extraction

Extraction of total RNA has been described previously (Chomczyski and Sacchi 1987). Briefly, myotubes grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and lysed directly on the dishes using 2 ml of solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 10 mM β-mercaptoethanol). The monolayer was scraped with a rubber policeman to ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform–isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12 000 *g*) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added. The samples were mixed and stored at –20°C overnight. RNA was collected by centrifugation (12 000 *g*, 20 min at 3°C). The supernatant was discarded and the RNA pellet was resuspended on 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at –20°C overnight. The RNA pellet was recovered by centrifugation at 12 000 *g* for 15 min at 4°C, washed twice with 70% ethanol and dried under a vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

Northern blot analysis

Total RNA samples (25 μg) were denatured at 55°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. RNA was transferred onto nylon membranes (GeneScreenPlus, DuPont/NEN) and immobilized by baking at 70°C for 40 min. Selenoprotein W cDNA and 16 S ribosomal RNA oligonucleotides (Giovannoni 1991) were labeled by using DIG Labeling Kit (Boehringer Mannheim Biochemica, Indianapolis, IN). The RNA membrane was hybridized and washed according to the procedures described in DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim Biochemica, Indianapolis, IN). Briefly, the membrane was prehybridized at 42°C for 1 h in hybridization buffer (50% formamide, 5 X SSC, 0.02% SDS, 0.1% *N*-lauroylsarcosine and 2% Blocking Reagent). After prehybridization, heat-denatured DIG labeled selenoprotein W cDNA probe was added to hybridization buffer and the membrane was hybridized at 42°C overnight. Next day, the membrane was washed in 0.5 X SSC with 0.1% SDS at room temperature. After washing briefly in Buffer 1 containing 0.3% Tween 20, the membrane was incubated in Buffer 2 for 30 min. Then anti-digoxigenin-AP conjugated antibody was diluted in Buffer 2 (1:5000) and the membrane was incubated in this buffer for 30 min. The unbound antibody conjugate was removed by washing the membrane in Buffer 1. The membrane was then equilibrated in Buffer 3 for 2 min and substrate solution was added. After incubation at 37°C for 5 min, the excess substrate was removed and the membrane was exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). Before hybridization to the

internal control probe, the membrane was rinsed thoroughly in water and then incubated in stripping buffer (50% formamide, 50 mM Tris-HCI [pH 8.0] and 1% SDS) at 68°C for 1 h to remove the probe. After rinsing with water and then in 2 X SSC, the membrane was used for hybridization of internal control probe (16 S rRNA oligonucleotides).

Statistical analysis

Data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) method for comparing groups (Steel and Torrie 1980). A significance level of 5% was adopted for all comparisons.

Results

In the preliminary experiment, L8 muscle cells were cultured in basal medium with 10% CS to reach confluence, after which the serum concentration of the medium was reduced to 2% to induce the differentiation of myotubes. These data (Figure 1, lanes 5–11) showed that selenoprotein W levels (Figure 1) and GPX activities (data not shown) decreased gradually during differentiation. Some of the 4-day differentiated myotubes were incubated with either 10^{-6} M (lane 14) or 10^{-7} M (lane 13) selenium as selenite for 3 days, and the selenoprotein W levels (Figure 1) and GPX activities (data not shown) increased after 3-day incubation in response to selenium supplementation. Using the standards in lanes 1 to 3, the selenoprotein W levels were calculated to be 92, 70, 48, 30, 5, 4, ND, ND, ND, 2 and 7 ng mg/protein, respectively, in lanes 4 to 14. The GPX activities (nmol NADPH oxidized per min per mg protein) in cells used in these lanes were 95, 106, 90, 73, 54, 43, 38, 32, 42, 64 and 60, respectively.

Figure 2 shows the specific binding of antibody to selenoprotein W in L8 muscle cells. The antibody clearly binds only to a single low-molecular weight protein (selenoprotein W). The effect of differentiation on selenoprotein W levels and GPX activities is shown in Table 1. These results indicated that selenoprotein W levels remained unchanged during cell differentiation ($P > 0.05$).

To further examine the long-term effect of different selenium concentrations on selenoprotein W, L8 muscle cells which had differentiated in medium were incubated with various concentrations of selenium as selenite for 6 days (Figure 3). Both selenoprotein W level and GPX activity reached the plateau where 10^{-7} M Se as selenite was added (Table 2). These data showed that selenoprotein W increased as selenium concentration (up to 10^{-7} M) in the medium was increased ($P < 0.05$).

Figure 4 gives the GPX activities of rat L8 muscle cell incubated with differentiation medium (basal medium with 2% CS) with additions of 10^{-6} M, 10^{-7} M or 10^{-8} M selenium (Figures 4A, 4B and 4C, respectively) as either selenite, selenocystine or

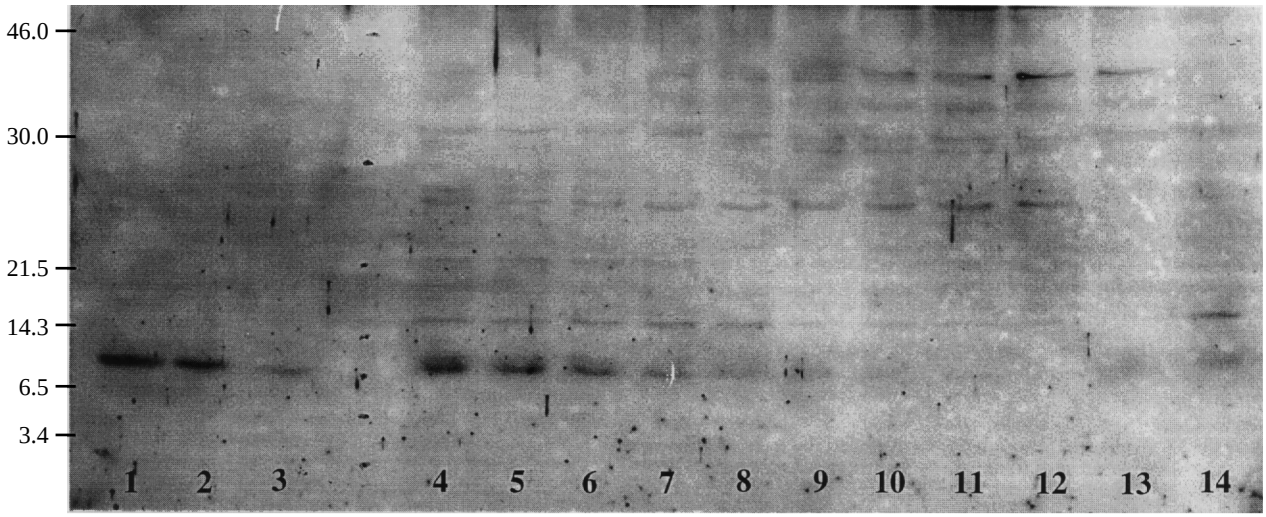

Figure 1. Western blot of selenoprotein W levels at various differentiation stages of rat L8 muscle cells. Lanes 1–3: Pure selenoprotein W (15 ng, 10 ng, 5 ng). Lane 4 is myoblast in basal media + 10% CS. Lanes 5–11 are 1-day, 2-day, 3-day, 4-day, 5-day, 6-day and 7-day differentiated myotubes in basal medium + 2% CS, respectively. Lanes 12–14 are 4-day differentiated myotubes incubated with different media (basal medium + 2% CS, basal medium + 2%CS + 10^{-7} M selenite, basal medium + 2% CS + 10^{-6} M selenite, respectively) for 3 days.

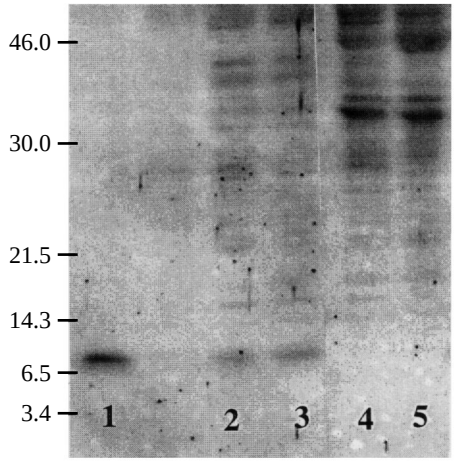

Figure 2. Western blot of selenoprotein W in L8 muscle cells. Rabbit polyclonal antibody against rat selenoprotein W peptide sequence was used in Western blot analysis. Lane 1 is pure selenoprotein W (5 ng) from rat muscle. Lanes 2 and 4 are L8 myoblasts. Lanes 3 and 5 are 1-day differentiated muscle cells. For Western blot assay, lanes 2 and 3 were incubated with primary antibody whereas lanes 4 and 5 were incubated with antibody which was preabsorbed (10 μg peptide per ml antibody solution) with synthetic peptide of selenoprotein W.

selenomethionine (Semet) for 16, 24 and 48 h. Similar patterns were noted when the myotubes were incubated with either 10^{-6} M (Figure 4A) or 10^{-7} M (Figure 4B) selenium as these three sources (Figure 4C). GPX activity at 16 h incubation did not differ from the initial values ($P > 0.05$). However, GPX activity increased to the greatest extent at 48 h incubation with selenite ($P < 0.05$), followed by selenocysteine and Semet. These results indicated that the availability of different selenium forms for GPX synthesis is sodium selenite ⩾ selenocysteine > Semet ($P < 0.05$).

Table 1. Effects of differentiation on selenoprotein W level and GPX activity in L8 muscle cells

Differentiated stage	Se-W level*	GPX activity*
Day 0	69 ± 20	82 ± 3[a]
Day 2	120 ± 20	104 ± 2[b]
Day 4	82 ± 16	85 ± 2[a,c]
Day 6	111 ± 18	90 ± 2[c]

*Values are expressed as mean ± S.E. Day 0 = beginning of differentiation. Day 6 = 6-day differentiation. Different superscripts denote significant difference. ($P < 0.05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized per min per mg protein.

L8 muscle cells were cultured and differentiated in basal medium with 10% CS.

Table 2. Effects of various selenium concentrations on selenoprotein W level and GPX activity in 6-day differentiated myotubes, as shown in Figure 3

Media	Se-W level*	GPX activity*
Differentiation medium (DM)**	20 ± 5[a]	83 ± 8[a]
DM + 10^{-8} M selenite	151 ± 27[b]	110 ± 5[b]
DM + 10^{-7} M selenite	714 ± 109[c]	149 ± 7[c]
DM + 10^{-6} M selenite	411 ± 94[d]	144 ± 9[c]

*Values are expressed as mean ± S.E. Different superscripts denote significant difference ($P < 0.05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized per min per mg protein.

L8 muscle cells were cultured in basal medium with 10% CS to reach confluence, and subsequently incubated in various media to differentiate for 6 days.

**Differentiation medium = basal medium + 2% CS.

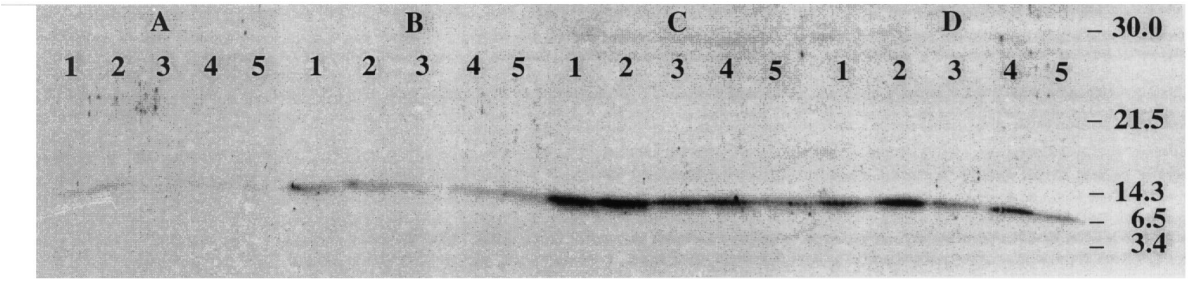

Figure 3. Western blot of selenoprotein W L8 myotubes cultured in differentiation medium supplemented with various selenium concentrations as selenite for 6 days. Rat L8 myoblasts were grown to reach confluence in basal medium + 10% CS; Differentiation medium was used to differentiate myoblasts to myotubes. Lanes A 1–5: differentiation medium (basal medium + 2% CS). Lanes B 1–5: differentiation medium + 10^{-8} M Se. Lanes C 1–5: differentiation medium + 10^{-7} M Se. Lanes D 1–5: differentiation medium + 10^{-6} M Se.

Effects of serum and selenium concentrations on GPX activity at various times are shown in Figure 5. The patterns of GPX activities in myotubes in response to selenium concentrations for 10^{-6} M (Figure 5A), 10^{-7} M (Figure 5B) and 10^{-8} M (Figure 5C) were similar. L8 myotubes cultured with basal medium remained constant, but there were increases, although erratic, when serum was added to basal medium ($P < 0.05$). When selenium was added to the incubation medium, GPX activity increased dramatically to the greatest extent at 48 h of incubation. There were no significant differences in GPX activities between myotubes incubated in basal medium with serum and differentiation medium with selenium ($P > 0.05$).

Figure 6 shows the effects of different forms of selenium at 10^{-6} M on selenoprotein W level during various incubation times. There were no significant differences among these three selenium forms at 16 (Figure 6A) and 24 h (Figure 6B) incubation ($P > 0.05$). After 48 h (Figure 6C) incubation, selenite appeared to be the most available form of selenium for muscle cell usage ($P < 0.05$). There were no differences between selenium forms as selenocystine and selenomethionine ($P > 0.05$). The availability of different selenium forms for selenoprotein W synthesis is sodium selenite > selenocystine = selenomethionine ($P < 0.05$).

Effects of different selenium concentrations and type of medium on selenoprotein W level at various times are shown in Figure 7. Addition of selenium in incubation medium caused an increase ($P < 0.05$) in selenoprotein W level (Figure 7), but there was no difference among myotubes incubated with various selenium concentrations at 16 h (Figure 7A). Selenium concentration as 10^{-6} M caused the highest selenoprotein W level ($P < 0.05$) at 24 h incubation (Figure 7B), whereas selenium concentrations of 10^{-6} M and 10^{-7} M gave similar results ($P > 0.05$) at 48 h incubation (Figure 7C).

Table 3 shows the effects of selenium at 10^{-6} M and serum on selenoprotein W levels in 6-day differentiated myotubes. Selenoprotein W level in myotubes incubated with basal medium plus either 10% CS or selenium was significantly higher than those in the differentiation medium ($P < 0.05$). Similar to selenoprotein W levels, GPX activity between myotubes incubated in basal medium with 10% CS and differentiation medium with selenium did not differ significantly ($P > 0.05$).

Table 4 shows the selenoprotein W level and GPX activity for myotubes in various media for 6 days. The selenoprotein W levels and GPX activities between myotubes incubated in basal medium

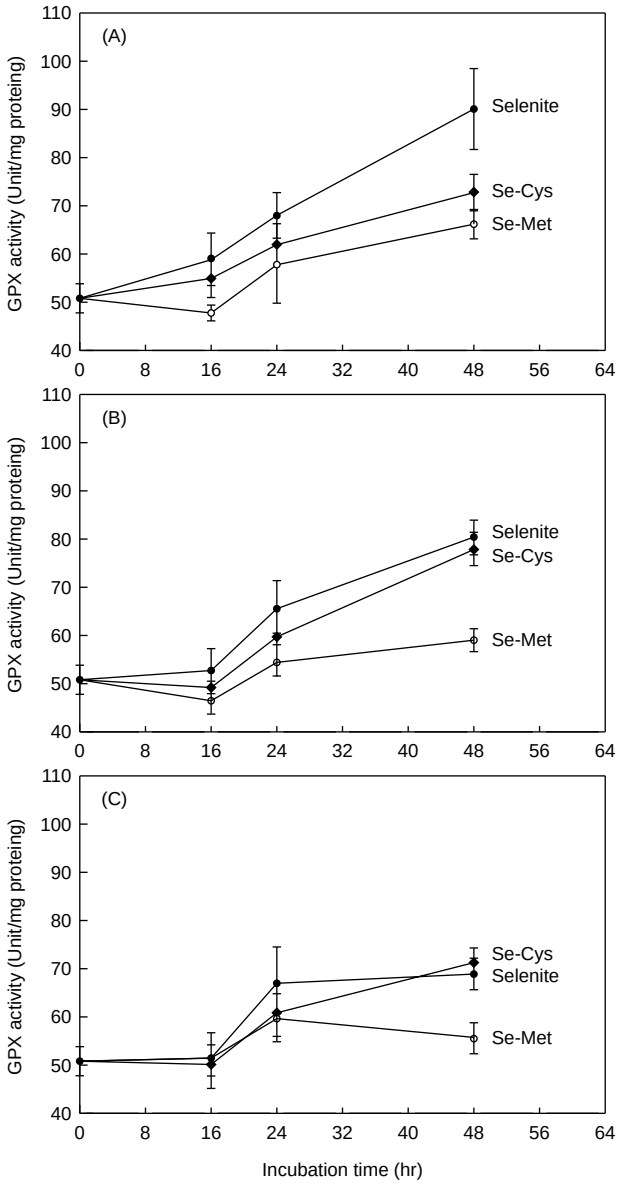

Figure 4. Effects of different forms of Se on GPX activities (nmol NADPH oxidized per min per mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 h). (A) differentiation medium (basal medium + 2% CS) with 10^{-6} M Se as selenite, L-selenomethionine or L-selenocysine; (B) differentiation medium with 10^{-7} M Se as selenite, L-selenomethionine or L-selenocystine; and (C) differentiation medium with 10^{-8} M Se as selenite, L-selenomethionine or L-selenocystine.

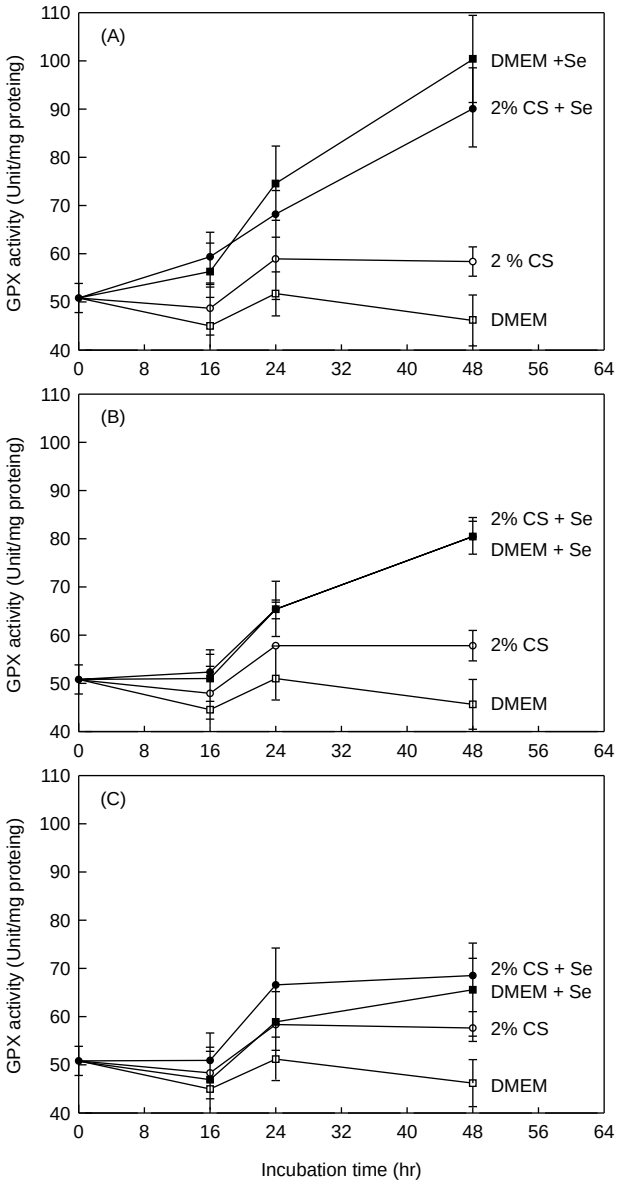

Figure 5. Effects of serum and selenium concentrations on GPX activities (nmol NADPH oxidized per min per mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 h). (A) basal medium (without serum, DMEM), basal medium with 10^{-6} M selenite (DMEM + Se), differentiation medium (2% CS), or differentiation medium with 10^{-6} M selenite (2% CS + Se); (B) basal medium (without serum, DMEM), basal medium with 10^{-6} M selenite (DMEM + Se), differentiation medium (2% CS), or differentiation medium with 10^{-7} M selenite (2% CS + Se); and (C) basal medium (without serum, DMEM), basal medium with 10^{-6} M selenite (DMEM + Se), differentiation medium (2% CS), or differentiation medium with 10^{-8} M selenite (2% CS + Se).

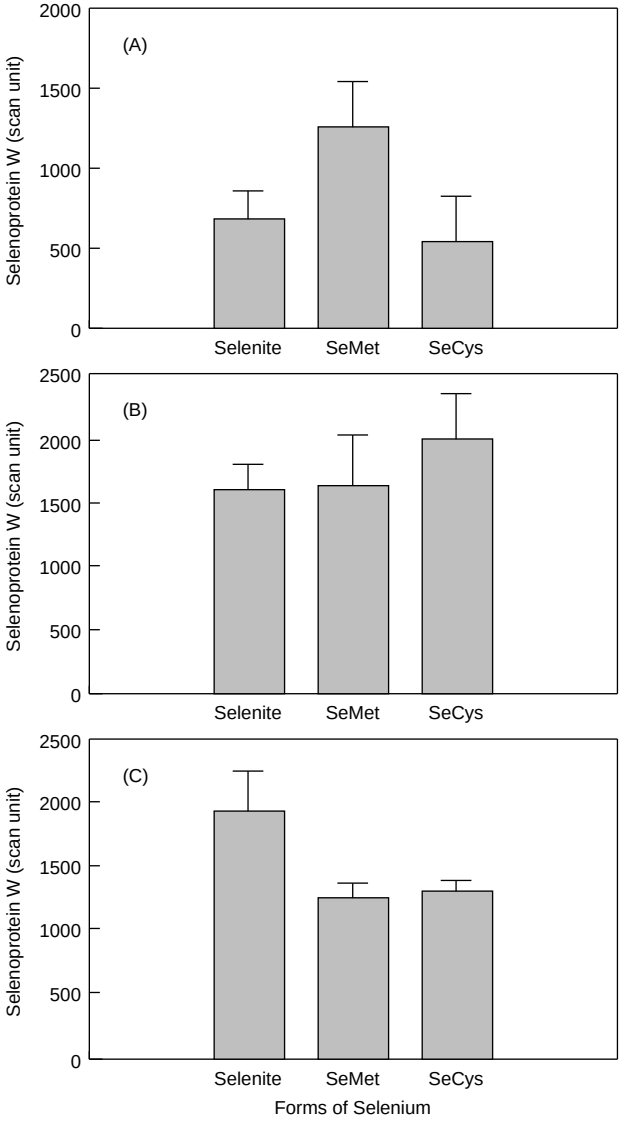

Figure 6. Effects of different forms of selenium (10^{-6} M of selenite, L-selenomethionine or L-selenocystine) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for (A) 16 h; (B) 24 h; and (C) 48 h. Different electrophoresis was performed for the various time periods, and thus the difference can only be compared within the same incubation time.

or differentiation medium with selenium were significantly higher ($P < 0.05$) than in media without addition of selenium. The selenoprotein W levels and GPX activity were similar in cells grown in basal medium and differentiation medium. The results of Tables 3 and 4 suggest that the reduced selenoprotein W level and GPX activity found in myotubes incubated with lower serum medium was due to the decreased selenium concentration which was provided by serum in the medium.

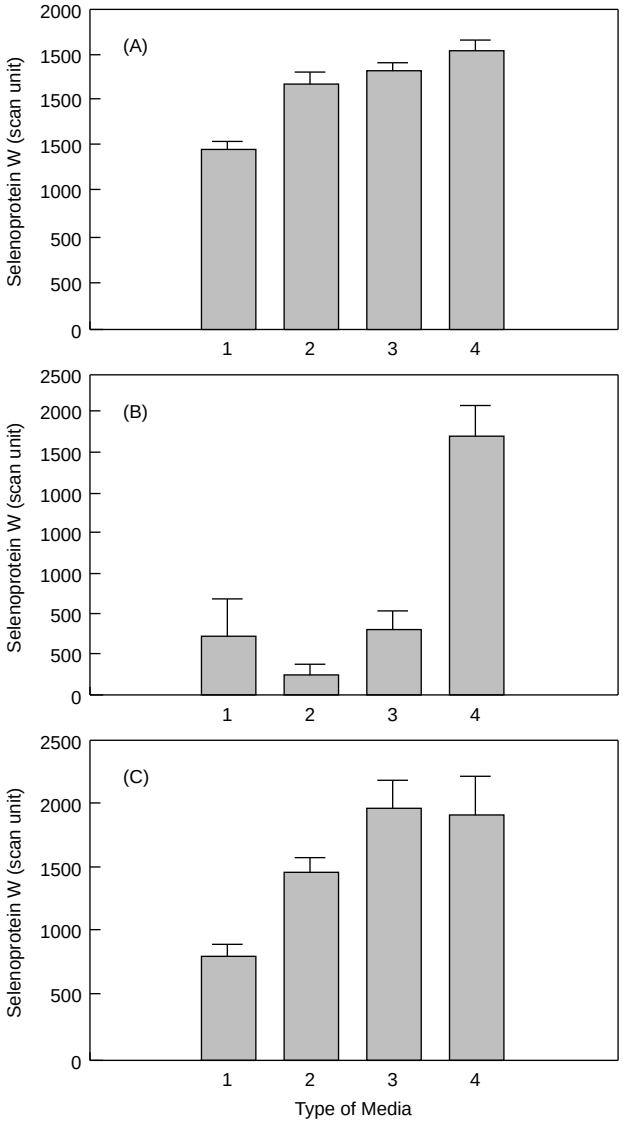

Figure 7. Effects of different selenium concentrations (10^{-8} M, 10^{-7} M or 10^{-6} M Se as selenite) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for (A) 16 h; (B) 24 h; and (C) 48 h. Bar 1: differentiation medium (basal medium + 2% CS); Bar 2: differentiation medium + 10^{-8} M Se; Bar 3: differentiation medium + 10^{-7} M Se; Bar 4: differentiation medium + 10^{-6} M Se. Different electrophoresis was performed for the various time periods, and thus the difference can only be compared within the same incubation time.

Northern blot analysis indicated that selenium influenced the mRNA level for selenoprotein W (Figure 8), based on the intensity of the bands. The intensity of the bands was greater when selenium was added (lanes 7–9) than when selenium was not added (lanes 4–6), indicating that this element affects the mRNA levels.

Table 3. Effects of serum and selenium concentrations on selenoprotein W level and GPX activity in 6-day differentiated L8 myotubes

Media	Se-W level*	GPX activity*
Basal medium + 10% CS	392 ± 49[a]	99 ± 2[a]
Differentiation medium (DM)**	141 ± 65[b]	83 ± 8[b]
DM + 10^{-6} M selenite	442 ± 116[a]	110 ± 5[a]

*Values are expressed as mean ± S.E. Different superscripts denote significant difference ($P < 0.05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized per min per mg protein.

L8 muscle cells were cultured in basal medium with 10% CS to reach confluence, then incubated in various media to differentiate for 6 days.

**Differentiation medium = basal medium + 2% CS.

The calculated results are shown in Table 5. There is no difference in 18 S rRNA levels among different media ($P > 0.05$). This was used as an internal control probe. The results indicated that selenoprotein W mRNA of L8 myotubes incubated in various selenium concentrations reached a plateau at concentration of 10^{-8} M in response to selenium.

Discussion

The L8 muscle cells were cultured in basal medium with 10% CS to reach confluence, and differentiation of myotubes was induced when the CS concentration was reduced to 2%. The decrease in selenoprotein W level during muscle cell differentiation

Table 4. Effects of serum and selenium on selenoprotein W levels and GPX activity in 6-day differentiated L8 myotubes

Media	GPX activity*	Se-W level* (scan unit)
Basal medium**	46 ± 5[a]	359±31[a]
Basal medium + 10^{-6} M selenite	100 ± 9[b]	1446±576[b]
Differentiation medium	58 ± 3[a]	210±26[a]
Differentiation medium + 10^{-6} M selenite	90 ± 8[b]	318±113[b]

*Values are expressed as mean ± S.E. Different superscripts denote significant difference ($P < 0.05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized per min per mg protein.

**Basal medium = without serum. Differentiation medium = basal medium + 2% CS.

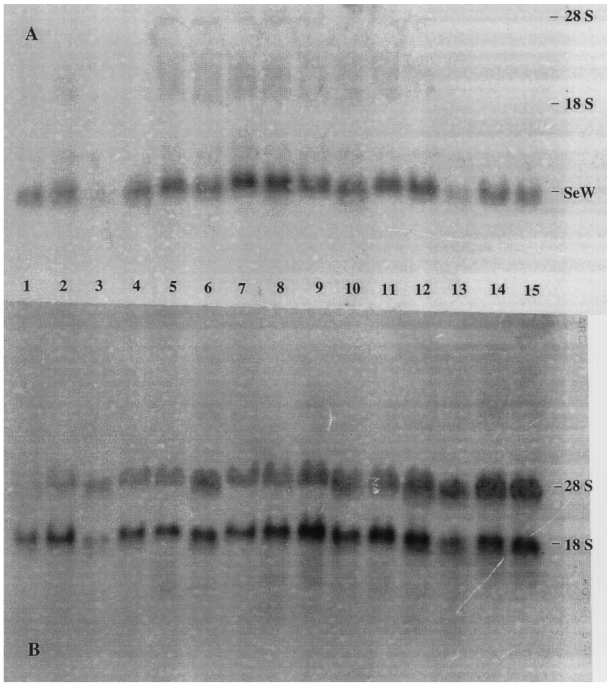

Figure 8. Northern blot analysis of total RNA from rat L8 myotubes. 4-Day differentiated myotubes were incubated in differentiation medium with additions of various concentrations of selenium as selenite for 48 h. A: hybridization with selenoprotein W cDNA probe. B: hybridization with internal control probe (16 S rRNA oligonucleotides). Lanes 1–3: initial control; lanes 4–6: differentiation medium (basal medium + 2% CS); lanes 7–9: differentiation medium + 10^{-6} M Se; lanes 10–12: differentiation medium + 10^{-7} M Se; lanes 13–15: differentiation medium + 10^{-8} M Se.

(Figure 1) was due to the lower selenium concentration in the medium. However, selenoprotein W level did not change during muscle cell differentiation (Table 1). In addition, selenoprotein W level and GPX activity in myotubes cultured in high serum medium (10% CS) were higher than those cultured in low serum medium (2% CS), whereas selenoprotein W level and GPX activity were similar in myotubes cultured in low serum medium with selenium addition (2% CS + 10^{-6} M Se) and high serum medium (Table 3). These data indicated that the decrease in selenoprotein W during differentiation was due to the lower selenium concentration which was provided by serum in the medium. Selenium is obviously involved in regulation of selenoprotein W in L8 muscle cells. Thus, L8 muscle cells can be used as a model to study the influence of selenium on selenoprotein W levels.

Table 5. Effects of various selenium concentrations on selenoprotein W mRNA level in 4-day differentiated L8 myotubes as shown in Figure 8.

Media	Se-W mRNA*	18 S rRNA*	Ratio*
Differentiation medium (DM)**	1684±121[ac]	3525±158	0.48±0.03[a]
DM + 10^{-8} M selenite	1367±133[a]	1803±537	0.81±0.10[b]
DM + 10^{-7} M selenite	2008±50[bc]	2169±103	0.93±0.02[b]
DM + 10^{-6} M selenite	2499±221[b]	3159±650	0.93±0.02[b]

*Values are expressed as mean ± S.E. Different superscripts denote significant difference ($P < 0.05$). Selenoprotein W mRNA and 18 S rRNA are expressed as scan units. Ratio = Se-W mRNA/18 S rRNA.

**Differentiation medium = basal medium + 2% CS.

Concentration of selenium in the medium regulated the level of selenoprotein W in L8 muscle cells (Figures 3 and 7; Table 2). When selenium was reduced in the medium, the selenoprotein W content decreased. When selenium was higher in the culture medium, the levels of this protein likewise increased. This is similar to the results obtained *in vivo*. Supplementation of diets with selenium for rats (Yeh *et al*. 1995) and sheep (Yeh *et al*. 1996) result in increased levels of selenoprotein W in tissues. Muscle is more responsive than other tissues and thus the selenoprotein W level increased to a greater extent in muscle upon selenium addition. Likewise, when selenium is limited in the diet, the levels of this selenoprotein are low. These results indicate that selenium regulates selenoprotein W both *in vivo* and *in vitro*, and indicate that *in vitro* results mimic the *in vivo* results.

In general, the pattern for GPX activity was similar to that for selenoprotein W levels in the present work. When this was studied in various tissues of sheep, there was no correlation between these two selenoproteins in various tissues examined, whereas the patterns for these two selenoproteins were similar in ovine muscle (Yeh *et al*. 1996). This suggests differential regulations of selenoprotein W and GPX among the various tissues. However, the regulation of these two selenoproteins appears to be similar in muscle both *in vivo* and *in vitro*.

Among the three different forms of selenium tested, selenite is the most available one for both selenoprotein W and GPX synthesis in L8 muscle cells, whereas Semet is the least effective form

(Figures 4 and 6). The reduced ability of L8 cells to utilize Semet for selenoprotein W and for GPX activity is in agreement with prior work on GPX induction (White and Hoekstra 1979; Beilstein and Whanger 1987; Karle *et al*. 1983). In the work with human lymphocytes, there was no correlation between uptake of selenium and induction of GPX activity. Selenocysteine was incorporated at three times the rate of selenite, and Semet at 15 times the rate of selenite. Thus, the lower activity of GPX with addition of Semet is not due to less uptake by the cells. This is likely due to the non-specific incorporation of Semet in place of methionine during selenoprotein synthesis (White and Hoekstra 1979). However, selenite was found to be more effective than selenomethionine for GPX induction in lymphocytes (Karle *et al*. 1983).

To verify the specific incorporation of selenocystine and Semet, L8 myotubes were incubated in medium with addition of cysteine or methionine for 48 h. However, selenoprotein W levels and GPX activities did not differ significantly ($P > 0.05$) between cells incubated with these amino acids (data not shown). These results indicate that the incorporation of selenocystine and Semet into selenoproteins in selenium-dependent.

It had been reported that only one-third of whole body selenium was used for GPX synthesis and two-thirds of whole body selenium was used for synthesis of other selenoproteins in rat tissues (Hawkes *et al.* 1985). Our data indicated that the response of selenoprotein W to selenium in muscle cells was more sensitive and rapid than was GPX. Selenoprotein W showed responses to selenium addition as low as 10^{-8} M for 16 h (Figure 7A), whereas GPX activity did not show differences until additions at 10^{-7} M selenium for 48 h were used (Figure 5B). This implies that the priority usage for selenium in medium is for selenoprotein W synthesis, followed by GPX synthesis in L8 muscle cells.

Among various selenium concentrations, 10^{-7} M selenium is the optimum concentration for maximum synthesis of selenoprotein W and GPX in L8 muscle cells (Figures 4, 5 and 7). This is consistent with work on GPX induction. Based on GPX activity, the selenium requirements of four cell types was 10^{-7} M when selenite was used as the selenium source (Beilstein & Whanger 1987; Beilstein *et al*. 1987). This is also consistent with growth patterns. Selenium requirements appeared to be maximum for growth at 10^{-7} M for human fibroblasts, mouse myoblasts and ovine fibroblasts, but Chang liver cells grew just as well on a basal medium, which contained 10^{-8} M selenium, as on the selenium-supplemented ones. This suggests that the selenium requirements vary between different cell types (Beilstein *et al*. 1987).

The results in our study indicated that selenium regulated selenoprotein W synthesis both in transcription and translation. Northern blot analysis showed that selenoprotein W mRNA increased to maximum at a selenium concentration of 10^{-8} M in cultured L8 myotubes for 48 h (Figure 8A; Table 5), whereas selenoprotein W level reached a plateau at a selenium concentration of 10^{-7} M (Figure 7). During selenoprotein synthesis, selenium is co-translationally inserted into selenoprotein sequence. However, in our study the results indicated that in addition to translational regulation, selenium also regulates selenoprotein W synthesis at the transcriptional level. This is consistent with the study in kidney cells which showed that mRNAs of GPX and 5′-DI were regulated by selenium concentration in culture media (Gross *et al*. 1995). Whether selenium regulates gene expression of selenoprotein W directly or indirectly still needs further investigation.

Selenoprotein W level in L8 myotubes incubated in basal medium (without serum) with addition of selenium for 48 h was significantly higher ($P < 0.05$) than that in myotubes incubated in low serum medium (2% CS) with addition of selenium (Table 3). This result implies that serum may have a negative effect. To investigate the possibility that serum protein may bind selenium and reduce its availability, differentiation medium with and without serum was used. Media with low and high CS were supplemented with three different concentrations (10^{-6} M, 10^{-7} M and 10^{-8} M) of selenium as selenite, and incubated at 37°C for 48 h. When trichloroacetic acid was added to precipitate proteins, the selenium content in the precipitate of differentiation medium increased as the serum concentration increased (data not shown). This implies that proteins in serum may be involved in binding the selenium and reduced its availability to the muscle cells.

Like selenoprotein P, the metabolic function of selenoprotein W is unknown. Selenium deficiency in lambs and calves results in white muscle disease, a disorder characterized by degeneration of both skeletal and cardiac muscles (Schubert *et al*. 1961). In humans, severe dietary selenium deprivation in discrete regions of China is associated with an endemic juvenile cardiomyopathy called Keshan disease (Chen *et al*. 1980). Muscle weakness in patients on long-term parenteral nutrition can be alleviated and prevented by selenium supplementation (Brown *et al*. 1986). Therefore, there is sufficient evidence to

indicate that selenium is important for normal muscle metabolism. Selenoprotein W is present in muscle at higher concentration than in other tissues, and in selenium deficient animals selenoprotein W level decreased dramatically in muscle (Yeh *et al*. 1996). It is not known whether selenoprotein W has any significant role in muscle metabolism, but its sensitive and rapid responses to selenium status indicates that it may play a role in metabolism in this tissue. Further studies to identify the possible function of selenoprotein W are necessary.

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