SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 25, No. 7, pp. 1864–1867, 2006
© 2006 SETAC
Printed in the USA
0730-7268/06 $12.00 + .00

Short Communication

ISOLATION AND PARTIAL CHARACTERIZATION OF PROTEINS INVOLVED IN MATERNAL TRANSFER OF SELENIUM IN THE WESTERN FENCE LIZARD (*SCELOPORUS OCCIDENTALIS*)

JASON M. UNRINE,*† BRIAN P. JACKSON,‡ WILLIAM A. HOPKINS,§ and CHRISTOPHER ROMANEK†‖
†Advanced Analytical Center for Environmental Sciences, Savannah River Ecology Laboratory, University of Georgia, P.O. Drawer E, Aiken, South Carolina 29802, USA
‡Departments of Chemistry and Earth Sciences, Dartmouth College, 6105 Fairchild Hall, Hanover, New Hampshire 03765, USA
§Wildlife Ecotoxicology & Physiological Ecology Program, Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, 100 Cheatham Hall, Blacksburg, Virginia 24061, USA
‖Department of Geology, University of Georgia, Geography–Geology Building, Athens, Georgia 30602, USA

(*Received* 23 *October* 2005; *Accepted* 16 *December* 2005)

Abstract—Selenium from dietary exposure is efficiently transferred from mother to offspring in oviparous vertebrates, where it can cause severe teratogenic effects. We isolated and partially characterized proteins involved in maternal transfer of selenium in the oviparous lizard *Sceloporus occidentalis* using size-exclusion chromatography, inductively coupled plasma–mass spectrometry, and polyacrylamide gel electrophoresis. Selenium from dietary selenomethionine exposure was incorporated into at least three egg proteins. One of these proteins was lipovitellin. The other two proteins may be part of a previously unknown mechanism of maternal transfer of Se that is independent of vitellogenesis or albumin secretion. Our results suggest at least three pathways for maternal transfer of Se in vertebrates that may vary in importance depending on the species.

Keywords—Maternal transfer Selenium *Sceloporus occidentalis* Inductively coupled plasma–mass spectrometry Size-exclusion chromatography

INTRODUCTION

Selenium is an essential trace element in vertebrates at sub-mg/kg dry mass dietary concentrations, but it can become a teratogen when dietary concentrations exceed a few mg/kg dry mass [1]. The primary anthropogenic sources of Se in the environment are irrigation drainage in areas of high geological Se abundance and aquatic disposal of coal combustion wastes. Selenium enters the environment primarily in inorganic forms, but it may undergo a variety of transformations in the environment. Inorganic Se may be taken up by plants, where it can be transformed to the seleno–amino acids selenomethionine and selenocysteine [2]. Evidence suggests that proteinaceous selenomethionine is the species most closely associated with adverse reproductive effects in oviparous wildlife [2]. Selenomethionine is believed to be one of the primary Se species trophically transferred from prey to vertebrate predators, and it is more toxic in the diet than inorganic forms of Se [2–4]. Documented effects of maternal transfer of Se following dietary selenomethionine exposure in birds include axial and limb malformations, eye malformations, reduced hatching weight, and reduced survival of offspring [4]. Very little is known about the mechanisms by which Se is transferred from mother to offspring in oviparous vertebrates. A previous study showed incorporation of Se into the yolk precursor vitellogenin, and the yolk proteins lipovitellin and phosvitin, in white sturgeon (*Acipenser transmontanus*) [5], whereas other studies showed Se incorporation in ovalbumin, conalbumin, globulin, ovomucoid, and flavoprotein in domestic chicken (*Gallus gallus*) egg albumin [6,7].

Vertebrate eggs vary considerably in their anatomical and biochemical composition, particularly among reptiles [8,9]. Elucidation of biochemical mechanisms involved in maternal transfer of Se may provide insights regarding interspecies differences in Se teratogenicity and lead to the identification of biochemical markers of exposure that are better predictors of reproductive toxicity compared with bulk ovarian or egg concentrations. The objectives of the present study were to determine the extent to which dietary selenomethionine is incorporated into lizard eggs and ovarian follicles in proteinaceous forms, to determine if Se is distributed among proteins strictly according to S content, and to isolate and characterize Se-containing proteins in eggs and follicles.

MATERIALS AND METHODS

Laboratory exposures and tissue sampling

Western fence lizards (*Sceloporus occidentalis*) were exposed to Se via a simulated food chain as described elsewhere [10]. The lizards used in the present study were from the F_1 generation of parental stock that originated from the San Joaquin Valley (CA, USA). Western fence lizards are particularly suitable as a laboratory model and have a geographic distribution that overlaps with areas in the western United States where Se contamination is of great concern. Although the present paper addresses only females, both male and female hatchling lizards were exposed and housed individually (n = 15 per sex per treatment) [10]. Selenium was first introduced into the live prey of lizards, the house cricket (*Acheta domestica*), by providing the prey with Se-enriched diets. Diets were formulated by grinding Iams Chunks® dog food (Dayton, OH, USA) into a fine powder, adding an appropriate amount of seleno-D,L-methionine (Sigma, St. Louis, MO, USA; Se treatments) or control amino acid solution (D,L-methionine; con-

* To whom correspondence may be addressed (unrine@srel.edu).

trols) to the food matrix and then drying the food at 40°C overnight. Selenium concentrations in crickets and lizard tissues were determined by inductively coupled plasma–dynamic reaction cell–mass spectrometry (ICP-DRC-MS; Elan DRC Plus; Perkin-Elmer, Norwalk, CT, USA). The resulting whole-body Se concentrations for *A. domestica* ranged from 14.81 to 16.00 μg/g. Lizards received rations of *A. domestica* equaling 30% of their body mass per week. After 98 d on their respective diets, all lizards were euthanized, and an egg or follicle, depending on which was present, was removed, flash-frozen in liquid N_2, and stored at −70°C. All lizards in both treatments survived to the end of the experiment and reached reproductive maturity (based on secondary sexual characteristics), and no treatment effect on growth or food consumption was observed [10]. Teratogenesis and reproductive output were not evaluated. This is consistent with a previous study in which no effects on growth, food consumption, or body condition were observed in brown house snakes (*Lamprophis fuliginosus*) exposed to dietary selenomethionine up to 20 μg/g [11]. It is important to note that teratogenesis and reproductive output are the most sensitive endpoints of Se toxicity in birds and fish [1,3]. Lizards accumulated an average of 14.03 μg Se/g dry mass in ovaries [10]. Most of the animals had not yet ovulated. Two eggs (lengths, 12.95 and 12.56 mm; widths, 8.04 and 7.46 mm; masses, 382.7 and 424.1 mg) were present in animals from the selenomethionine treatment, and one egg was present in an animal from the control treatment (length, 13.44 mm; width, 7.44 mm; mass, 323.5 mg). All eggs were used for subsequent analyses along with three randomly selected follicles from the selenomethionine treatment with a diameter of 7.98 ± 0.33 mm (mean ± standard error of the mean) and a mass of 304.4 ± 8.3 mg and two randomly selected follicles from the control treatment with diameters of 8.32 and 8.53 mm and masses of 307.8 and 321.7 mg.

Chromatography and mass spectrometry

Whole eggs or follicles were homogenized in four volumes of ice-cold 50 mM Tris-HCl (pH 7.2) containing 0.1% Triton X-100 in prechilled glass microtissue grinders. The homogenates were then centrifuged at 1,200 *g* for 20 min at 4°C. The supernatants were decanted and analyzed without further preparation. The pellets were then lyophilized and, along with small aliquots of the supernatant, were digested in HNO_3 using a MARS 5 microwave digestion system (CEM, Mathews, NC, USA). The digests were analyzed by ICP-DRC-MS to determine how much Se was recovered by the extraction process. Recovery of Se from follicles and eggs from the Se-exposed group was 20 ± 4%, which is in the range of what is expected in aqueous extractions and also reflects the low solubility of yolk platelets [12,13]

Proteins in the supernatants were separated on a Superdex™ 10/30 200HR size-exclusion chromatography (SEC) column (Amersham Biosciences, Piscataway, NJ, USA) with an effective size-separation range of 10 to 600 kDa and a bed volume of 24 ml. The mobile phase was 100 mM Tris-HCl (pH 7.2), and the flow rate was held constant at 0.5 ml/min. Absorbance was quantified at 210 nm using an inline ultraviolet (UV) absorbance detector (model 460; Alltech, Deerfield, IL, USA). The eluate from the SEC column was introduced into the ICP-DRC-MS using a concentric nebulizer and a Peltier-cooled cyclonic spray chamber (Elemental Scientific, Omaha, NE, USA). The DRC was pressurized with 5% H in Ar at a flow rate of 0.4 ml/min. The low-mass cutoff parameter (RPq) was set at 0.45. Use of the DRC combined with the cooled spray chamber suppressed $^{40}Ar^{40}Ar$, $^{16}O^{18}O$, and $^{16}O^{15}N$ interferences, allowing for quantification of ^{80}Se, ^{34}S, and ^{31}P, respectively. The element-specific chromatograms were collected in data-only mode with Se, S, Zn, and P monitored at *m/z* 80, 34, 66, and 31, respectively, in peak hopping mode with a dwell time of 100 ms/reading. The number of readings was adjusted so that the replicate measurement time spanned the entire chromatographic separation. Sulfur was recorded to determine if Se distribution reflected the normal distribution of S in proteins. Zinc and P were used to help identify the fractions that were likely to contain the yolk proteins lipovitellin and phosvitin [14,15]. Fractions (0.5 ml) also were collected from subsequent SEC separations performed under identical conditions for further characterization of Se-containing peaks. The SEC column, which separates based on hydrodynamic radius, was calibrated from 12.4 to 669 kDa using a molecular marker kit for SEC obtained from Sigma. This kit contained a number of metalloproteins that were detected both by ICP-MS and UV absorbance. The excluded and total volumes (V_o and V_t, respectively) were determined using blue dextran (2,000 kDa) and acetone (58 Da).

Electrophoresis

Protein subunit molecular weight was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Aliquots of individual fractions from each Se-containing peak were diluted 1:1 in Laemmli buffer (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, and 5% β-mercaptoethanol; pH 6.8) and heated to 100°C for 5 min. Twenty microliters of the diluted samples were loaded onto polyacrylamide gels for electrophoresis. Electrophoresis was performed with either 4% stacking/5% resolving or 4% stacking/15% resolving, 0.375 M Tris-HCl (pH 8.8), precast gels (format, 6.8 × 8.6 cm) using 25 mM Tris, 192 mM glycine, and 0.1% SDS running buffer (pH 8.3). The gels were run with constant potential (200 V) and variable current, starting at 60 mA and ending around 10 mA, for approximately 45 min. After electrophoresis, gels were stained with Biosafe™ Coomassie (Bio-Rad, Hercules, CA, USA). Molecular weights were calibrated against a set recombinant protein standards ranging in molecular weight from 10 to 250 kDa. All electrophoresis reagents and standards were obtained from Bio-Rad.

RESULTS

At least three major Se-containing peaks were present in the SEC chromatographs, with a relatively small amount of Se eluting near the V_t of the column (21.7 ml) (Fig. 1). The first major Se peak in the chromatograph, hereafter referred to as P-1, also was enriched in P and had an estimated molecular weight of 263 kDa. Two additional, incompletely resolved peaks, P-2 and P-3, also were present, with estimated molecular weights of 69 and 46 kDa, respectively. Further analysis of fractions by SDS-PAGE revealed that the unresolved peaks, P-2 and P-3, in fact consisted of two proteins, whereas peak P-1 consisted of one protein (Fig. 2). Protein P-1 was a homodimer with a subunit molecular weight of 111 kDa (Fig. 2A). Protein P-2 was a 57-kDa monomer, whereas P-3 was a heterodimer with subunit molecular weights of 24 and 23 kDa (Fig. 2B). Protein P-1 also contained large quantities of Zn and P (Fig. 1B). Protein P-1 was present in eggs, and in trace amounts in one follicle but not in the others.

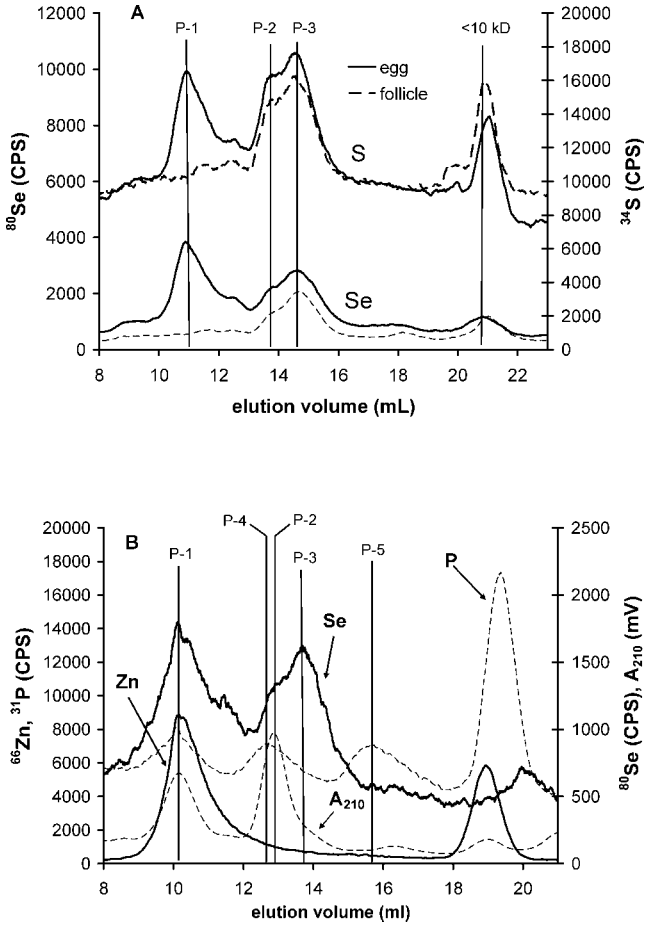

Fig. 1. Size-exclusion chromatographs of egg and follicle protein extracts from *Sceloporus occidentalis* exposed to selenomethionine via a laboratory food chain. Inductively coupled plasma–dynamic reaction cell–mass spectrometry was used for detection of Se and S (**A**) and of Zn and P (**B**). Ultraviolet absorbance at 210 nm also is shown for eggs (**B**), as are example chromatographs for eggs and follicles (**A**) and for eggs only (**B**). CPS = counts per second.

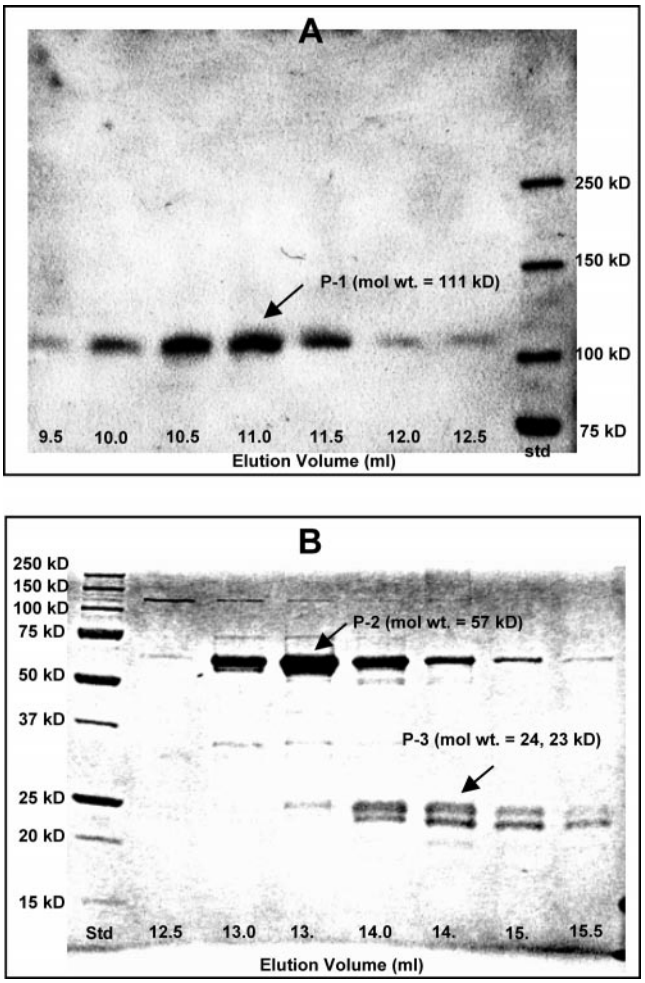

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of Se-containing egg proteins from *Sceloporus occidentalis* exposed to selenomethionine via a laboratory food chain. Protein P-1 (**A**) is a homodimer with a subunit molecular weight of 111 kDa. Protein P-2 (**B**) is a monomer with a molecular weight of 57 kDa. Protein P-3 (**B**) is a heterodimer with subunit molecular weights of 23 and 24 kDa.

Proteins P-3 and P-4 were always present in both eggs and follicles.

Two additional phosphoproteins, P-4 and P-5, were identified that did not coelute with any of the Se-containing proteins (Fig. 1B). Phosphoprotein P-4 had an estimated molecular weight of 75 kDa, and phosphoprotein P-5 had an estimated molecular weight of 23 kDa. Neither P-4 nor P-5 was stained by Biosafe Coomassie after SDS-PAGE.

All the aforementioned proteins were present in the control follicles and egg; however, the available control egg was less developed than the Se-exposed eggs and contained less protein P-1. All these proteins were enriched in the same heteroatoms (S, P, and Zn) as their Se-containing counterparts but did not have detectable Se.

DISCUSSION

We isolated several Se-containing proteins that likely play important roles in transference of Se from mother to offspring in *S. occidentalis*. The distribution of Se in egg proteins mirrored the distribution of S, which is consistent with steric replacement of S with Se in synthesis of S-containing amino acids and/or replacement of methionine and cysteine with selenomethionine and selenocysteine during charging of methionyl or cysteinyl tRNA by tRNA synthases [16]. Very little Se was present in the low-molecular-weight fraction that eluted near the V_t, indicating that most egg Se is present in proteinaceous form rather than in small molecules.

The evidence suggests that P-1 is lipovitellin. Protein P-1 is similar in subunit molecular weight (111 kDa) to a lipovitellin found in the lizard *Anolis pulchellus* (112 kDa) and the lizard *Podarcis sicula* (110 kDa) [8,15]. Furthermore, it migrated as a single band under nonreducing conditions with an estimated molecular weight of 263 kDa, which is similar to lipovitellin in *A. pulchellus* (molecular wt, 223 kDa) [15]. The presence of Zn and P further support the conclusion that P-1 is lipovitellin, because it is a Zn-binding phosphoprotein [13–15].

The phosphoproteins P-4 and P-5 were probably phosvitins and contained little or no Se or S. The molecular weight of P-4 (75 kDa) is the same as that of a phosvitin found in the lizard *A. pulchellus* (75 kDa), and P-5 (23 kDa) is similar in molecular weight to a phosvitin found in the lizard *P. sicula* (29 kDa) [8]. Proteins P-4 and P-5 could not be stained with Coomassie, which, combined with their P content and lack of UV absorbance, supports the conclusion that they are phosvitins [8]. This differs from the result of a previous study [5],

in which Se was incorporated into phosvitin in the white sturgeon (*A. transmontanus*). This may result from the fact that the S content of phosvitins varies among species. For example, phosvitin contains few of the S-bearing amino acids methionine and cysteine in *Xenopus laevis* or *G. gallus* but are relatively enriched in these amino acids in *Oncorhynchus mykiss* [5,13,17]. We did not observe significant quantities of intact vitellogenin, as observed in *A. transmontanus*, because it does not accumulate in lizard follicles or eggs as it does in fish [15].

The identities of P-2 and P-3 are unknown; however, P-2 (69 kDa) may be similar to proteins identified in *A. pulchellus*, designated as D-5 and D-6, both of which have molecular weights of 62 kDa, but isoelectric points of 4.5 and 4.0, respectively [15]. A ladder pattern was present in the lanes on SDS-PAGE gels containing high concentrations of P-2 (Fig. 2B), consistent with what is sometimes observed for acidic proteins. Morales et al. [15] hypothesized that these acidic, 62-kDa proteins may be similar to a group of water-soluble yolk proteins found in chicken eggs known as livetins, which are similar to serum albumin. The components of P-3 (molecular wts, 24 and 23 kDa) may be similar to neutral heterodimers present in *A. pulchellus* eggs that have subunit molecular weights of 19 and 21 kDa, designated as D-1 and D-2 [15]. Reptilian egg proteins have not been well characterized (particularly in squamates) [8]; therefore, the function of these proteins is not known [15].

The present results, taken together with those of previous studies outlined below, suggest that multiple biochemical pathways for maternal transfer of Se exist that differ across major vertebrate groups. Vitellogenin is synthesized in the liver and transported to the ovary in the blood, where it enters the developing follicle by receptor-mediated endocytosis and is processed further by enzymatic cleavage to lipovitellin and phosvitin, the two major vertebrate yolk proteins [8]. Vitellogenin, lipovitellin, and phosvitin were enriched in Se in white sturgeon [5]. We have shown that this is only true for the lipovitellin component of vitellogenin in *S. occidentalis*. Ovalbumins have been shown to contain Se in eggs from common hens [7]. Ovalbumins, which are secreted by the oviduct, are rich in cysteine and methionine [9,18] and, therefore, are vectors for maternal transfer of Se. Squamate reptiles generally do not secrete an albumin layer, as do birds, crocodilians, and turtles [9]. This suggests that in fish, amphibians, and squamate reptiles, Se may be transported through serum to the egg from the liver as vitellogenin, whereas in birds, crocodilians, and turtles, additional oviductal contributions of Se occur postovulation.

The present study demonstrated the existence of one or two previously unknown mechanisms of maternal transfer of Se that are unrelated to vitellogenesis or albumin secretion. Selenium-containing egg proteins (P-2 and P-3) were present in *S. occidentalis* that were not derived from vitellogenin or oviductal secretions, as evidenced by their lack of P content and presence in the follicles before ovulation. These proteins are most likely the product of previously unknown mechanisms involved in maternal transfer of Se. Further characterization of these proteins may lead to an enhanced mechanistic understanding of maternal transfer of Se in oviparous vertebrates and, ultimately, better predictions of potential impacts of Se on wildlife populations.

Acknowledgement—The authors thank J. Baionno, B. Staub, L. Talent, K. Selcer, and two anonymous reviewers. The present research was supported by the Environmental Remediation Sciences Division of the Office of Biological and Environmental Research, U.S. Department of Energy, through the Financial Assistant Award DE-FC09-96SR18546 to the University of Georgia Research Foundation.

REFERENCES

1. Lemly AD. 1998. Pathology of selenium poisoning in fish. In Frankenberger WT, Engberg RA, eds, *Environmental Chemistry of Selenium.* Marcel Dekker, New York, NY, USA, pp 281–296.
2. Fan TW-M, Teh SJ, Hinton DE, Higashi RM. 2002. Selenium biotransformations into proteinaceous forms by food web organisms of selenium-laden drainage waters in California. *Aquat Toxicol* 57:65–84.
3. Ohlendorf HM. 2003. Ecotoxicology of selenium. In Hoffman DJ, Rattner BA, Burton GA, Cairns J, eds, *Handbook of Ecotoxicology.* Lewis, Boca Raton, FL, USA, pp 465–501.
4. Heinz G, Hoffman D, Krynitsky A, Weller D. 1987. Reproduction in mallards fed selenium. *Environ Toxicol Chem* 6:423–433.
5. Kroll KJ, Doroshov SI. 1991. Vitellogenin: Potential vehicle for selenium bioaccumulation in oocytes of the white sturgeon (*Acipenser transmontanus*). In Williot P, ed, *Acipenser*. Cemagref Publishers, Antony, France, pp 99–106.
6. Davis RH, Fear J. 1996. Incorporation of selenium into egg proteins from dietary selenite. *Br Poult Sci* 37:197–211.
7. Jacobs K, Shen L, Benemariya H, Deelstra H. 1993. Selenium distribution in egg white proteins. *Z Lebensm Unters Forsch* 196: 236–238.
8. Romano M, Rosnova P, Anteo C, Limatola E. 2004. Vertebrate yolk proteins: A review. *Mol Reprod Dev* 69:109–116.
9. Blackburn DG. 1998. Structure, function and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. *J Exp Zool* 282:560–617.
10. Hopkins WA, Staub BP, Baionno JA, Jackson BP, Talent LG. 2005. Transfer of selenium from prey to predators in a simulated terrestrial food chain. *Environ Pollut* 134:447–456.
11. Hopkins WA, Staub BP, Baionno JA, Jackson BP, Roe JH, Ford NB. 2004. Trophic and maternal transfer of selenium in brown house snakes (*Lamprophis fuliginosus*). *Ecotoxicol Environ Saf* 58:285–293.
12. Daun C, Lundh T, Onning G, Akesson B. 2004. Separation of soluble selenium compounds in muscle from seven animal species using size exclusion chromatography and inductively coupled plasma mass spectrometry. *J Anal At Spectrom* 19:129–134.
13. Wallace RA. 1970. Studies on amphibian yolk: IV. An analysis of the main-body component of yolk platelets. *Biochem Biophys Acta* 74:505–518.
14. Montorzi M, Falchuck KH, Vallee BL. 1995. Vitellogenin and lipovitellin: Zinc proteins of *Xenopus laevis* oocytes. *Biochemistry* 34:10851–10858.
15. Morales MH, Valles AM, Baerga-Santini C. 1987. Studies of the egg proteins of tropical lizards: Purification and partial characterization of yolk proteins of *Anolis pulchellus*. *Comp Biochem Physiol B* 87:125–136.
16. Moroder L. 2005. Isosteric replacement of sulfur with other chalcogens in peptides and proteins. *J Pept Sci* 11:187–214.
17. Byrne BM, van het Schip AD, van de Klundert JA, Arnberg AC, Gruber M, Geert AB. 1984. Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene. *Biochemistry* 23:4275–4279.
18. Stein PE, Leslie AG, Finch JT, Carrell RW. 1991. Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J Mol Biol* 221: 941.