Fresenius J Anal Chem (2000) 367 : 96–98 – © Springer-Verlag 2000

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Direct determination of selenoproteins in polyvinylidene difluoride membranes by electrothermal atomic absorption spectrometry

Received: 28 June 1999 / Revised: 14 September 1999 / Accepted: 16 September 1999

Abstract A method for the direct determination of selenoproteins in plastic membranes after protein separation by gel electrophoresis was developed. Quantification was based on the determination of the selenium content of the proteins by electrothermal atomic absorption spectrometry (ET-AAS) after manual introduction of membrane pieces into the graphite furnace. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry electroblotting. After staining the membrane, the protein bands were excised and chemical modifier was added on top of the excised membrane prior to atomic absorption measurement. Acceptable linearity was achieved in the range 2–10 ng Se, corresponding to selenium concentrations close to 1 mg/L, when aqueous solutions of selenomethionine standard as well as selenoprotein standard were applied to the membrane. A characteristic mass of 54 ± 4 pg/0.0044 s was obtained for the selenoprotein standard. Protein transfer from polyacrylamide gel to the membrane was quantitative and no interferences were introduced. The method was used for identification of selenoprotein P after enrichment of the protein from human plasma.

Introduction

Identification of proteins after gel electrophoresis is normally done by staining of the gel for sensitive but non-specific detection of proteins. When the proteins are immobilized on a membrane by electroblotting and stained with immuno-chemical techniques, a more sensitive and specific staining can be obtained. The quantitative information from these staining procedures are usually obtained by comparison of the band intensities of standards and sample. However, for specific staining of a protein, a specific antibody to the protein must be available.

In human plasma, selenium mainly appears covalently bound into proteins as selenocysteine and selenomethionine. Selenomethionine is incorporated non-specifically in place of methionine whereas selenocysteine is incorporated specifically. Only selenocysteine containing proteins seem to have a selenium related function. So far, three selenium containing proteins are identified in human plasma [1]. The extracellular glutathione peroxidase (GSH-Px) and selenoprotein P which both contain selenocysteine and albumin in which selenomethionine is incorporated non-specifically instead of methionine. GSH-Px consists of 4 identical subunits with an M_r of ~23,000, each subunit contains 1 selenocysteine residue [2]. The human selenoprotein P cDNA has been sequenced and the deduced amino acid sequence contains 10 selenocysteines [3]. In rat and in human exist at least two isoforms [4, 5]: One full length isoform and one isoform with the same N-terminal but C-terminal truncated [4]. The full length isoform contains 10 selenocysteines and has a mobility in SDS-PAGE close to an apparent M_r of 60,000, whereas the truncated isoform contains only 1 selenocysteine and has a mobility in SDS-PAGE close to an apparent M_r of 55,000 [4, 5].

Selenoproteins can be analyzed by separation of the proteins by capillary electrophoresis followed by detection with inductively coupled plasma mass spectrometry [6]. This method demands expensive equipment and is still not a routine technique. Separation of the proteins by SDS-PAGE, and subsequent laborious wet ashing of the excised protein bands is an other possibility.

The aim of the present study was to develop a fast and reliable method for identification and quantification of selenium in protein bands after SDS-PAGE analysis of enriched selenoprotein samples.

Experimental

SDS-PAGE was run in 10% pre-cast tris-glycine polyacrylamide gels from Novex (Frankfurt/Main, Germany) according to the recommendation of the manufacturer and stained with Coomassie R-350 (Pharmacia, Uppsala, Sweden). Semi-dry electroblotting was performed from SDS-PAGE gels onto PVDF-membranes using the Hoefer TE70 semi-dry transfer unit (Hoefer, San Francisco, CA, USA) according to Matsudaira [7].

A 100 mg L^{-1} Se stock solution of selenomethionine (Sigma, St. Louis, MO, USA) was prepared in 0.14 M HNO_3. A 1 mg L^{-1} Se selenomethionine standard was prepared by dilution with water. A GSH-Px standard containing 1 mg L^{-1} Se was prepared by dissolving bovine GSH-Px (Sigma, St. Louis, MO, USA) in water. The solution gave a single band in SDS-PAGE when visualized by staining with Coomassie. Selenoprotein P was purified from human plasma by immobilized cobalt affinity chromatography followed by heparin affinity chromatography [8].

All non-specified chemicals were of analytical grade from either Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Purified water from a Milli-Q de-ionization unit (Millipore, Bedford, MA, USA) was used throughout.

The integrated atomic absorption was determined using a Perkin Elmer Zeeman 5000 AAS equipped with a HGA-500 graphite furnace and pyrolytic graphite tubes without platforms. The instrumental settings are given in Table 1.

Procedure. Selenium standards were pipetted in amounts of 2–10 ng onto the PVDF-membrane (0.5 cm × 1 cm). The membrane was dried for 10 min at 60 °C, then 5 µL of a palladium modifier containing 4 g L^{-1} Pd in 1.2 M nitric acid was added and the membrane was dried at 60 °C for another 10 min. Alternatively one volume of selenomethionine standard was mixed with one volume of chemical modifier and this mixture was pipetted onto the PVDF-membrane, which was dried for 10 min at 60 °C. The membrane was placed in the graphite furnace and the atomic absorbance was measured.

Selenoproteins were analyzed as described above or after SDS-PAGE electrophoresis. The gels were electroblotted onto a PVDF-membrane. After staining with Coomassie R-350, the relevant bands were cut out from the membrane and 5 µL of chemical modifier added.

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Table 1 Temperature program used for the determination of selenium by ET-AAS. Argon was used as the purge gas and oxygen as the alternative gas. The measurements were done with a electrodeless discharge lamp at 196.1 nm with a lamp current of 10 mA and a slit width of 0.7 nm

Step	1	2	3	4	5	6	7	8	9
Temperature (°C)	130	160	600	600	600	1200	2500	2600	20
Ramp time (s)	50	35	20	0	0	10	0	1	1
Hold time (s)	5	15	0	15	20	40	5	5	10
Internal gas flow (mL/min)	300	300	300	0	300	300	0	300	300
Alternative gas flow (mL/min)				30					
Recorder							–5		
Read							–1		

Results and discussion

After direct pipetting of selenomethionine standards onto the PVDF membrane acceptable linearity was obtained in the range 2–10 ng selenium, corresponding to a concentration of selenium close to 1 mg/L. The data for the calibration curve is shown in Table 2. An acceptable correlation was achieved (r = 0.9971) and the characteristic mass of 52 pg is in the same order of magnitude as previously obtained for aqueous solutions of selenium [9]. Thus, no serious interferences were observed in the tested concentration range, and the PVDF-membrane was completely ashed during the temperature program.

If the selenomethionine standard and the chemical modifier were mixed prior to application on the PVDF membrane, the slope of the calibration curve was 50% higher, yielding a lower characteristic mass; the data are shown in Table 2. This difference may reflect an inadequate protection of selenium from premature volatilization during thermal pre-treatment when modifier and sample were not mixed in solution. It is, however, not possible to mix chemical modifier and the selenium species in solution, when using excised protein bands, and the mixing of selenium standards and chemical modifier in solution is therefore not compatible with the present method. The results also exclude the possibility of using simple aqueous standards directly applied into the graphite tube, instead of application of standards to the PVDF-membrane, for preparation of the calibration curve.

To examine the recovery of the transfer from polyacrylamide gel to PVDF-membrane, a calibration curve obtained by pipetting GSH-Px directly onto the membrane was compared with a calibration curve obtained by electroblotting identical amounts of GSH-Px onto the membrane. The results are shown in Table 2. The calibration curves have similar slopes and the fitted parameters are identical within the experimental error. Thus, the transfer of GSH-Px from the gel onto the PVDF-membrane is quantitative, and the blotting and the staining process do not introduce any new interferences that affect the determination of selenium in the tested concentration range.

It is not possible to assess the accuracy as certified reference materials are not available for selenoproteins. If a standard addition method should be used, a selenium species exactly identical to the analyte must be added, otherwise the added selenium species would be separated from the analyte by the electrophoresis step and therefore not included in the ET-AAS analysis of the excised band. However, as the slope for the GSH-Px calibration curve is identical with the slope of the selenomethionine calibration curve, selenomethionine standards applied directly on the PVDF-membrane can be used for calibration in the quantitative determination of selenium in a Coomassie stained protein band from a PVDF-membrane.

The limit of detection (LOD) of selenium was 1–2 ng per piece of membrane, in the case of GSH-Px this corresponds to 0.4 μg protein. The LOD for protein staining by Coomassie is 0.3–1.0 μg protein. Thus, when the protein is visible by Coomassie staining the selenium content can be determined with the present technique.

The method was applied to the analysis of a purified preparation of selenoprotein P from human plasma. After SDS-PAGE, electroblotting onto a PVDF-membrane and Coomassie staining, four bands were observed on the PVDF membrane. The bands were analyzed by the present method and two bands with apparent molecular weights of 55.000 and 60.000 were shown to contain selenium, whereas the other two bands did not contain selenium. The two bands containing selenium were identified as selenoprotein P by amino acid sequencing, whereas the other two bands were identified as non-selenoproteins.

The present method could probably be applied to the analysis of other metallo-proteins. However, only metallo-proteins containing a tightly bound metal ion will probably resist the harsh sample preparation before SDS-PAGE. In selenoproteins, selenium is covalently bound in the protein and therefore

Table 2 Statistical data for linear fits to selenium calibration curves in the range between 2–10 ng selenium

Selenium species Method[a]	Selenomethionine A	Selenomethionine B	GSH-Px A	GSH-Px C
Slope (s/ng)	0.084 ± 0.002	0.123 ± 0.009	0.081 ± 0.006	0.087 ± 0.005
Characteristic mass (pg/0.0044 s)	52 ± 1	36 ± 3	54 ± 4	51 ± 3
Intercept (s)	0.00 ± 0.01	0.00 ± 0.05	0.06 ± 0.04	–0.02 ± 0.03
r	0.9971	0.9903	0.9901	0.9942
LOD[b] (ng Se)	0.82	1.8	1.8	1.4

[a] A: The selenium standard was pipetted onto the membrane and after subsequent drying the chemical modifier was pipetted on the membrane. B: The selenium standard and the chemical modifier was mixed before pipetting on the membrane. C: GSH-Px was transferred from the SDS-PAGE gel to the membrane by electroblotting and the modifier was pipetted on the membrane afterwards

[b] Calculated as: LOD = $3 \times (S_{yx}/\text{slope})$

not lost during sample treatment. But if native PAGE, i.e. without boiling the protein in a SDS containing buffer, is used instead of SDS-PAGE it is much more likely that the metal ion stays bound in the protein.

Conclusion

ET-AAS after manual sample introduction can be used for identification and semi-quantitative determination of selenoproteins from PVDF-membranes after electroblotting from polyacrylamide gels.

Acknowledgements We wish to thank Dr. Anders Johnsen from the University Hospital, Copenhagen, for performing the amino acid sequencing.

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Fresenius J Anal Chem (2000) 367 : 98–100 – © Springer-Verlag 2000

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An enzyme linked immunoassay for the determination of deoxynivalenol in wheat based on chicken egg yolk antibodies

Received: 5 July 1999 / Revised: 29 November 1999 / Accepted: 2 December 1999

Abstract An indirect competitive enzyme linked immunoassay (ELISA) for the detection of the *Fusarium* mycotoxin deoxynivalenol (DON) in wheat was developed. Instead of the much more common antibody isolation from mammal serum, DON specific antibodies were, for the first time, isolated from the eggs of previously immunized hens. The limit of detection was 2 μg/L for standard curves and spiked wheat extracts. Recoveries for naturally contaminated samples (200–525 μg/kg) were between 80 and 125% compared with GC-ECD data. Concentrations for naturally contaminated samples were chosen with regard to current Austrian guidelines concerning DON levels in produce intended for human consumption, recommending a maximum of 500 μg DON/kg.

Introduction

DON (12,13-epoxy-3α,7α,15-trihydroxy-trichothec-9-ene-8-one) has been known to be produced by numerous *Fusarium* species, i.e. *F. culmorum, F. graminearum, F. roseum, F. sporotrichioides* and *F. sambucinum* [1–3]. It has been found as a contaminant in barley, maize, oat, rye, rice and wheat [4]. DON can interfere with both protein and DNA synthesis [5], the latter effect having been observed in cultured mammalian cells as well as the animals themselves [6–8]. Symptoms exhibited by animals afflicted with trichothecene toxicoses include vomiting, feed refusal, diarrhea and haemorrhage of intestines and muscles. DON also possesses immunosuppressive properties resulting in decreased resistance to microbial infections [9]. Commonly used methods for the determination of DON include GC-ECD [10], HPLC and immunoassays based on either polyclonal antibodies from mammal serum or monoclonal antibodies [11, 12]. Advantages of egg yolk antibodies (IgY) include resistance against acid, heat [13], repeated freezing and thawing [14] as well as high yield [15] and less influence on animal welfare [16], thus representing a viable alternative. So far, immunoassays based on IgY from chicken egg yolk have been employed for the determination of a number of mycotoxins, such as ochratoxin A [17], aflatoxin [18] and zearalenone [19]. The pursuit of further research in this field is also encouraged by the European Centre for the Validation of Alternative Methods (ECVAM), as these methods make an important contribution to animal welfare, also meeting scientific and commercial requirements [20].

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Experimental

Materials. DON standards were purchased from Sigma, rabbit anti-chicken-IgY horseradish peroxidase conjugate from Margaritella. Microtiter plates (Costar, 96 well flat bottom, high binding certified) were read on a TECAN SLT Spectra Instruments MTP reader (Grödig, Austria).

Synthesis of hapten-conjugates. Hapten conjugates were kindly provided by a cooperation partner who synthesized them as follows. DON was converted to 15-acetyl-DON according to Grove *et al.* [21] and subsequently conjugated to keyhole limpet hemocyanine and bovine serum albumin by the method of Sinha *et al.* [12]. For structures of DON and conjugates see Fig. 1; the –NH- group originates from a primary amino group of the protein.