Phytochemical Approach and Bioanalytical Strategy to Develop Chaperone-Based Medications

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Abstract: Currently, no pharmaceuticals for the etiological treatment of degenerative protein-misfolding diseases (e.g., ALS, Alzheimer's or prion diseases) are commercially available. Therefore, in this technical note theoretical considerations and practical approaches concerning the development of chaperone-based medications from medicinal plants (e.g., *Ginkgo biloba*) are reviewed and discussed in detail. Phytochaperones and other agents isolated from medicinal plants are proposed to serve as the general basis of drug development in protein-misfolding diseases.

Keywords: Phytochaperones, biofluids, Alzheimer's disease, medicinal plant extracts, CCS, *Ginkgo biloba*, SOD, molecular farming, metal cofactors, homeostasis, QPNC-PAGE.

INTRODUCTION

"Nature is the only book, which offers large contents on all leaves" (Johann Wolfgang von Goethe, 1749-1832). This citation of the famous poet and natural scientist reflects that natural sources as plants should be consulted in order to find answers to problems concerning human health and welfare. Medicinal plants, for example, may be an important option for developing drugs from natural bioorganisms for the treatment of different diseases.

Despite enormous economical, technical and scientific progress in the past years, fundamental developments for the accomplishment of important challenges of mankind concerning the effective treatment of several progressive degenerative and neurodegenerative diseases (e.g., ALS, Alzheimer's or Parkinson's diseases, et c.) are in its infacy. Many of them belong to the so-called "protein-misfolding diseases". Furthermore, the pharmaceutical industries are not interested in developing certain innovative drugs to defeat these debilitating disorders because existing drugs, the so-called "blockbusters" may be more profitable than taking a chance on improving public health [1].

For these reasons the time has come to leave the conventional ways of thinking behind and to give unconventional ideas a chance to be realized, e.g., by developing medicines for common and rare diseases by non-profit laboratories.

Furthermore, standardized analytical procedures (e.g., quantitative preparative native continuous polyacrylamide gel electrophoresis = QPNC-PAGE) as well as modern biomolecular approaches (e.g., nuclear magnetic resonance = NMR or matrix-assisted laser desorption ionization time-of-flight mass spectrometry = MALDI-TOF-MS), successfully applied for identifying and isolating bioactive (metallo-) proteins or enzymes in complex protein mixtures could make an essential contribution to the development of innovative drugs from medicinal plants. Other important scientific fields like molecular biotechnology may contribute to produce a sufficient quantity of medicines for all people suffering from the mentioned diseases by using certain molecular farming procedures.

It is a well-known fact that chemical and pharmacological chaperones have been found to be effective in preventing the misfolding of different disease-causing proteins, however, many of the compounds are highly toxic, reveal a lack of specificity or other unknown mechanisms of action in vivo. This technical note is an attempt to introduce a new class of pharmacologically active proteins, namely the (metallo-) phytochaperones, proposed to be the key molecules for the etiological treatment of protein-misfolding diseases (PMD), and to motivate medicinal, pharmaceutical and biochemical researchers worldwide to apply the methodological approaches presented in this study for developing chaperone-based medications.

MEDICINAL PLANTS AND PROTEIN-MISFOLDING DISEASES

Drug discovery from medicinal plants is a challenging field because it involves a multifaceted approach combining botanical, phytochemical, biological, and analytical techniques [2 - 37]. Well-known plants used in Traditional Chinese Medicine (TCM), Japanese, Ayurvedic and European Medicine relevant to the management of Morbus Alzheimer and other cognitive disorders are listed in Table 1 [2, 17, 18, 26, 32]. For example, standardized plant extracts from green leaves of the Ginkgo biloba tree are generally accepted in the treatment of AD [3, 4, 10, 17, 20, 32]. Through the antioxidant properties of its flavonoids these extracts may be able to protect hippocampal cells against toxic effects induced by amyloid ß (Aß) peptides [3]. An increase in the activity of the antioxidant enzymes, catalase and superoxide dismutase were further observed in rats treated with EGb 761 Ginkgo extract [4]. Another plant used in the Ayurvedic

medicine termed Bacopa monniera reduces Aß deposits in brain of AD animal model [8].

AD and many other neurodegenerative diseases are associated with disturbances of metal ion metabolisms and oxidative stresses postulated to be a downstream effect of abnormal Aß - metal ion interactions [7, 15, 21, 22, 25, 27]. Therefore, the metal ion homeostasis in a cell has to be regulated strictly by metallochaperones and other biomolecules (e.g., metallothioneins). For example, copper chaperones for superoxide dismutase (CCS) are essential metalloproteins for protecting and guiding copper ions to superoxide dismutase (SOD). Via specific protein-protein interactions SOD is activated by incorporating a Cu^{+} ion. As properly folded SOD molecules are very important antioxidants, these metal species contribute to a decreasing oxidative stress in cells [20, 21]. Therefore, metal chelation and antioxidants may be a potential therapy against neurodegenerative diseases [6, 7, 10, 15, 25]. Novel therapeutic strategies for the treatment of PMD are introduced by a deep insight review of Rochet [33].

Despite several therapeutic approaches, no preventive measure and effective treatment for PMD, especially Alzheimer's disease, is currently available [27]. Furthermore, vast majorities of psychoactive drugs are not natural products or are not derived from bioactive constituents of medicinal plants [26]. Therefore, some researchers demand to use natural plant extracts as possible protective agents of brain aging [2] and dementia therapy [32].

Plant extracts are multicomponent mixtures consisting of the bioactive main ingredients and secondary plant compounds which may interact with each other in a synergistic manner [9, 13, 20]. Drying and storing of medicinal plants are critical steps in the production processes of natural extracts and phytomedicines because the chemical stability of the bioactive ingredients may be adversely affected by the formation of unwanted artefacts [5]. As nature is the best combinatorial chemist and possibly has answers to all diseases of mankind [18] it is assumed that pharmacologically active ingredients in addition to the well-known plant flavonoids and terpenoids, namely proteins and enzymes, could be isolated and identified in medicinal plants for the effective treatment of several PMD. According to Table 1. especially extracts from the green leaves of Ginkgo biloba and other plants may contain bioactive metalloproteins for the treatment of AD or other degenerative disorders.

A majority of PMD are being considered caused primarily due to the imbalance between pro-oxidant and antioxidant homeostasis

[35]. An ideal therapeutic drug to dissolve Aß amyloid in AD, for example, would involve a compound selective for Cu^{1+}, Zn^{2+} and Fe^{3+}, but does not sequester Mg^{2+} and Ca^{2+} [7]. For example, Cu chaperones are a ubiquitous class of proteins that play a significant role in both Cu delivery and cellular protection against copper exposure under normal metabolic conditions by delivering and binding metal ions [16, 29]. Therefore, bioactive Cu chaperones may be the basis for developing novel lead molecules in the treatment of PMD.

It is a well-known fact that improperly folded CCS may play an important role in the etiology of Alzheimer's disease and other PMD. Therefore, the dysregulation of metal ion homeostasis and severe oxidative stresses in bioorganisms may occur [21, 22]. Furthermore, under non-physiological conditions a reduced enzyme activity of SOD and apo-SOD (apoenzymes) can be detected and quantified in blood of animals [37]. The apoenzymes are referred to as unfolded molecules. Therefore, it may be a necessary and helpful therapeutic approach to balance the metal ion homeostasis by activating unfolded SOD in blood of diseased bioorganisms. For these purposes, exogenous plant copper chaperones for SOD (pCCS) isolated from medicinal plants may be the lead molecules for an effective treatment of PMD. The pCCS activators may be able to recover the balance between pro-oxidant and antioxidant homeostasis of bioorganisms by copper ion transfer affecting the mechanism and speed of folding for the rapid achievement of the bioactive 3-D conformation of human SOD (hSOD), and by binding uncomplexed metal ions (e.g., Cu, Zn or Fe) in blood or other biofluids of living organisms.

DEVELOPMENT OF CHAPERONE-BASED MEDICATIONS

For developing chaperone-based medications from medicinal plants the following procedures could be very promising. For these purposes, properly and improperly folded copper cofactor-containing chaperones for superoxide dismutase present in blood samples of AD patients and probands have to be purified by innovative methods such as preparative native gel permeation chromatography (GPC) and QPNC-PAGE. The isolated metalloproteins of interest may be further elucidated by solution NMR spectroscopy. By applying an inductively coupled plasma mass spectrometry (ICP-MS) detection method for biometals, Fe, Cu, and Zn cofactors can be identified and quantified in the respective GPC and PAGE fractions. After electrophoretic separation improperly folded and bioactive metallochaperone proteins present in diseased or healthy blood can be resolved in the electrophergrams due to their different

isoelectric points [19, 21, 22]. Bioactive and inactive metalloproteins can also be isolated and quantified in other organisms, e.g., model plants by using the same methods [21-24].

Therefore, these efficient methods may be applied successfully in the discovery of pharmacologically-active metallochaperone proteins in medicinal plants as presented in Fig. (1). In this figure the basic investigations of selected protein-protein interactions and metalloprotein detection procedures in complex biological systems are schematically presented.

By incubating clinical biofluids (e.g., whole blood) with medicinal plant extracts (e.g., Ginkgo biloba), specific apoenzymes in a pathological blood sample (apo-SOD) might fold into their native conformation due to specific protein-protein interactions. Human SOD is a biomacromolecule with a molecular mass of about 32 kDa and might interact with the investigated plant CCS provided that pCCS has a similar molecular mass and structure and function compared to human CCS. The respective physiological effects can be studied using the proposed methods of the workflow schemes according to Fig. (1).

Plant extracts may be obtained by homogenising leaves of medicinal plants in liquid nitrogen by using mortars and pestles. The pulverized samples may be stored above liquid nitrogen or subsequently, extracted using a buffer solution. Medicinal plant extracts (e.g., Ginkgo biloba) are prepared under non-denaturing conditions by using a physiological buffer (e.g., 20 mM Tris-HCl, 1 mM NaN_3, pH 7.2). Plant material and buffer solution may be homogenised in a ratio of 1:10 (m/m). After centrifugation of the plant homogenate the resulting supernatant is used for merging plant extract and blood. The incubation time is extended to a maximum of about 15 to 60 minutes at 4° C to avoid uncontrolled proteolytic processes, protein precipitation and destabilization of metal cofactor-containing proteins in this very complex system consisting of plant and human matrices. Hereafter, an aliquot of the protein mixture is chromatographed on a Sephadex G-50 SF column. Only a very small elution range (MW 30 kDa for globular proteins) according to the void volume of the GPC method used is relevant for isolating some specific metal cofactor-containing proteins, CCS and SOD, by using QPNC-PAGE. The respective GPC conditions recommended for separating high molecular mass protein fractions are exemplary presented in Fig. (2). The complementary QPNC-PAGE parameters have already been listed in various articles or protocols [21-24, 34].

After GPC, a fraction of the void volume with the highest Cu concentration is separated by QPNC-PAGE. As result, physiological amounts of properly folded hSOD and pCCS may be isolated in a few specific PAGE fractions. Furthermore, the respective Cu cofactors of these biomolecules may be detected as "twin peaks" in the resulted electropherogram by using ICP-MS. The ratio of peak areas of the copper species may indicate that certain medicinal plants may contain bioactive pCCS or not because in untreated blood of AD patients the hSOD and hCCS peaks may be very small (low Cu concentrations) while in incubated blood samples the peak areas involving hSOD molecules may rise in a QPNC-PAGE electropherogram corresponding to high Cu concentrations after a PAGE run.

In order to develop chaperone-based medications from medicinal plants highly purified pCCS may be further isolated, elucidated and identified after the QPNC-PAGE run using a combination of 2-D PAGE (2-DE) followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and Bioinformatics presented in Fig. (3). The limitations of current proteomics technologies as referred to MALDI-TOF-MS and 2-DE are reviewed in [14]. An approach for identifying a high molecular mass metal protein in the model plant Arabidopsis thaliana by using these efficient methods is exemplary presented in [36].

The genetic code of the identified bioactive metallochaperone proteins in medicinal plants is a prerequisite for producing genetically modified plants (e.g., Nicotiana tabacum), designed to express the pCCS from medicinal plants as presented in Fig. (3). For the effective production of bioactive pCCS so-called "molecular farming" approaches are involved. Molecular farming is a challenging, new and promising technique using transgenic plants to produce foreign proteins as pharmaceutical ingredients. For these purposes plant growth and metabolism has
to be optimized under standardized conditions in order to maximize protein concentrations in roots and shoots [30]. It is important to mention that this method is an alternative to the microbial expression systems enabling the correct folding of recombinant proteins [12, 31]. Of course, other clinical and pharmaceutical approaches as already described in literature [2 – 37] have to be used for development of chaperone-based medications.

CONCLUSIONS

In this technical note different approaches including the molecular biotechnology, molecular farming, biology and analytical chemistry are proposed to be the initial steps for

developing chaperone-based medications from medicinal plants. Plant copper chaperones for superoxide dismutase (pCCS) extracted from natural bioorganisms are urgently needed for the etiological treatment of protein-misfolding diseases (e.g., ALS, Alzheimer's or prion diseases) because pCCS may have the ability to activate human apo-superoxide dismutase (hSOD) in biofluids. The interrelationships between pCCS and hSOD in human beings and animals are essential for recovering the metal ion homeostasis and balance between pro-oxidative and antioxidative processes in the cells of these organisms. Therefore, this approach could help to prevent abnormal protein-misfolding processes and subsequent oxidative stresses occuring in bioorganisms.

In addition to the well-known medicinal plants, e.g., Ginkgo biloba, other "living fossils" should be evaluated with respect to chelating and antioxidative properties in cells concerning the effective treatment of protein-misfolding diseases. For example, giant trees known as California Redwoods (e.g., Sequoia sempervirens), might be the natural sources of active metallochaperones or other important metal species.

REFERENCES

[1] Harvard Medical School. http://lansbury.bwh.harvard.edu/Files/2003_11_16.pdf (Accessed Dec 09, **2007**).
[2] Balunas, M.J.; Kinghorn, A.D. *Life Sci.*, **2005**, *78*, 431.
[3] Bastianetto, S.; Quirion, R. *Neurobiol. Aging*, **2002**, *23*, 891.
[4] Bridi, R.; Crossetti, F.P.; Steffen, V.M.; Henriques, A.T. *Phytother. Res.*, **2001,** *15*, 449.
[5] Carle, R.; Glenk, H.-G.; Gomaa, K.; Mueller, H. *Pharm. Unserer Zeit*, **1993**, *22*, 79.
[6] Chaudhuri, T.K.; Paul, S. *FEBS J.*, **2006**, *273*, 1331.
[7] Cuajungco, M.P.; Faget, K.Y.; Huang, X.; Tanzi, R.E.; Bush, A.I. *Ann. N.Y. Acad. Sci.*, **2000**, *920*, 292.
[8] Dhanasekaran, M.; Tharakan B.; Holcomb, L.A.; Hitt, A.R.; Young, K.A.; Manyam, B.V. *Phytother. Res.*, **2007**, *21*, 965.
[9] Eder, M.; Mehnert, W. *Pharm. Unserer Zeit*, **2000**, *29*, 377.
[10] Elsabagh, S.; Hartley, D.E.; Ali, O.; Williamson, E.M.; File, S.E. *Psychopharmacology*, **2005**, *179*, 437.
[11] Estrada, L.D.; Yowtak, J.; Soto, C. *Methods Mol. Biol.*, **2006**, *340*, 277.
[12] Faye, L.; Boulaflous, A.; Benchabane, M.; Gomord, V.; Michaud, D. *Vaccine*, **2005**, *23*, 1770.
[13] Gaedke, F. *Pharm. Unserer Zeit*, **2003**, *32*, 192.
[14] Garbis, S.; Lubec, G.; Fountoulakis, M. *J. Chromatogr. A*, **2005**, *1077*, 1.
[15] Hajieva, P.; Behl, C. *Curr. Pharm. Design*, **2006**, *12*, 699.
[16] Harrison, M.D.; Jones, C.E.; Dameron, C.T. *J. Biol. Inorg. Chem.*, **1999**, *4*, 145.
[17] Howes, M.-J.R.; Perry, N.S.L.; Houghton, P.J. *Phytother. Res.*, **2003**, *17*, 1.
[18] Jachak, S.M.; Saklani, A. *Curr. Sci.*, **2007**, *92*, 1251.
[19] Jensen, M.R.; Hass, M.A.S.; Hansen, D.F.; Led, J.J. *Cell. Mol. Life Sci.*, **2007**, *64*, 1085.
[20] Juretzek, W.; Mueller, W.E. *Pharm. Unserer Zeit*, **2002**, *31*, 370.
[21] Kastenholz, B. *Protein Pept. Lett.*, **2007**, *14*, 389.
[22] Kastenholz, B. *Protein Pept. Lett.*, **2006**, *13*, 503.
[23] Kastenholz, B. *Electroanalysis*, **2006**, *18*, 103.
[24] Kastenholz, B. *Anal. Lett.*, **2004**, *37*, 657.
[25] Kulkarni, P.P.; She, Y.M.; Smith, S.D.; Roberts, E.A.; Sarkar, B. *Chem.-Eur. J.*, **2006**, *12*, 2410.
[26] Kumar, V. *Phytother. Res.*, **2006**, *20*, 1023.
[27] Liu, G.; Huang, W.; Moir, R.D.; Vanderburg, C.R.; Lai, B.; Peng, Z.; Tanzi, R.E.; Rogers, J.T.; Huang, X. *J. Struct. Biol.*, **2006**, *155*, 45.
[28] Liu, J.; Henkel, T. *Curr. Med. Chem.*, **2002**, *9*, 1483.
[29] Mira, H.; Vilar, M.; Perez-Paya, E.; Penarrubia, L. *Biochem. J.*, **2001**, *357*, 545.
[30] Nagel, K.A.; Kastenholz, B.; Walter, A.; Gilmer, F.; Schurr, U. *Plant Biotech. J.*, **2008**, *00*, 000.
[31] Obermeyer, G.; Gehwolf, R.; Sebesta, W.; Hamilton, N.; Gadermaier, G.; Ferreira, F.; Commandeur, U.; Fischer, R.; Bentrup, F.-W. *Methods*, **2004**, *32*, 235.
[32] Perry, E.K.; Pickering, A.T.; Wang, W.W.; Houghton, P.J.; Perry, N.S.L. *J. Pharm. Pharmacol.*, **1999**, *51*, 527.
[33] Rochet, J.-C. *Expert Rev. Mol. Med.*, **2007**, *9*, 1.

[34] The Hebrew University of Jerusalem.
http://wolfson.huji.ac.il/purification/PDF/PAGE_SDS/QPNC-PAGE/QPNC_PAGEProtocol.pdf (Accessed Dec 09, **2007**).
[35] Tiwari, A.K. *Curr. Sci.*, **2001**, *81*, 1179.
[36] University of Bonn. http://hss.ulb.uni
-bonn.de/diss_online/math_nat_fak/2006/muktiono_budi (Accessed Dec 09, **2007**).
[37] West, E.C.; Prohaska, J.R. *Exp. Biol. Med.*, **2004**, *229*, 756.

Table 1. Traditional medicinal plants used for cognitive disorders

Medicinal Plant	Uses, Pharmaceutical and Clinical Effects
Centella asiatica L	Strengthens nervous function and memory, enhancement of cholinergic activity and thus, cognitive function.
Ginkgo biloba L	Improvement of memory loss associated with blood circulation abnormalties, favourable effects on neuronal cell metabolism, antioxidant activity, neuroprotective against β-amyloid toxicity *in vitro*.
Melissa officinalis L	Treatment of depression, hysteria and nervous insomnia, shows antioxidant effects.
Polygala tenuifolia Willd	Used in TCM as a cardiotonic and cerebrotonic, as a sedative and tranquillizer, and for amnesia, forgetfulness, neuritis, nightmares and insomnia.
Salvia lavandulaefolia Vahl. *Salvia officinalis* L	Cholinesterase inhibition, antioxidant and oestrogenic activities *in vitro*.
Salvia miltiorrhiza Bung	Treatment of blood circulation disorders, insomnia, neurasthenia and alleviation of inflammation.
Withania somnifera (L) Dun	Important herb in Ayurvedic medicine, treatment of inflammatory conditions, such as arthritis.

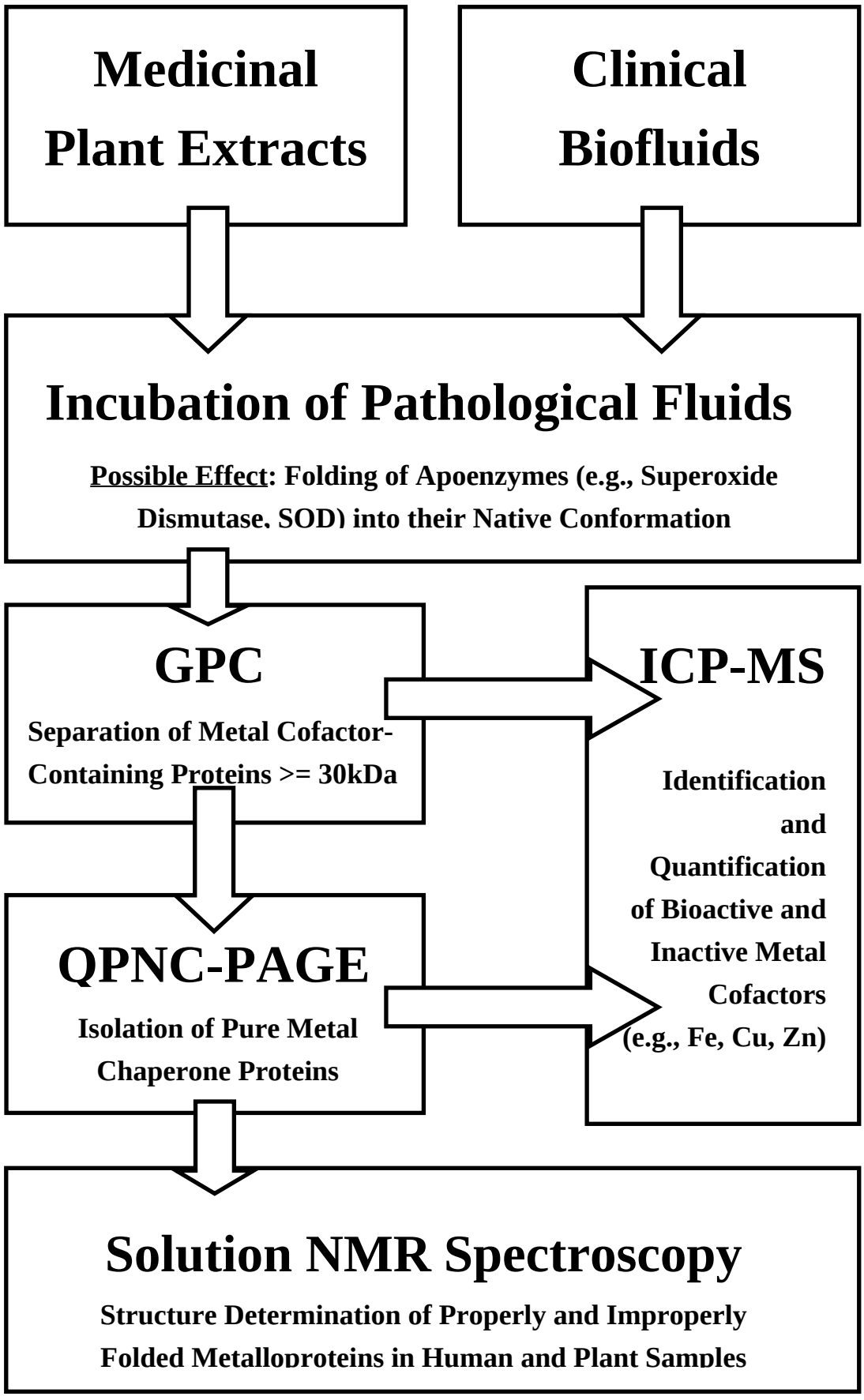

Fig. (1). Workflow schemes in metalloproteomics and interactomics.

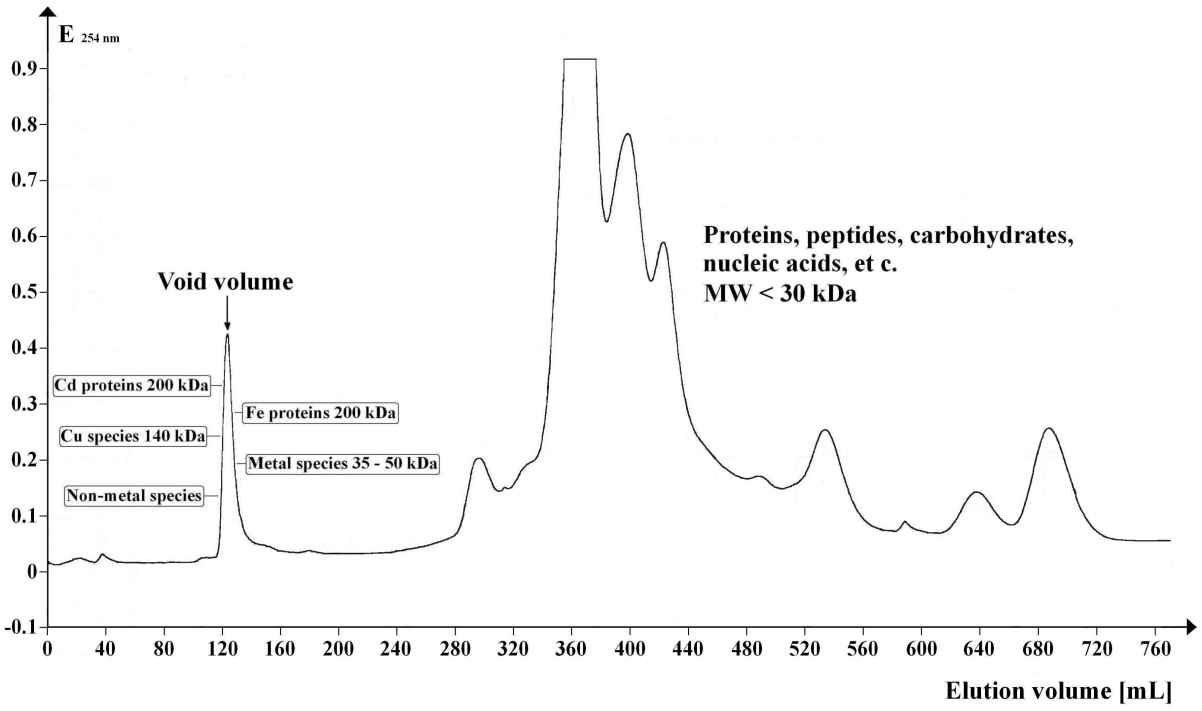

Fig. (2). Chromatogram showing the UV absorption profile of Arabidopsis supernatant separated on Sephadex G-50 Superfine. Gel volume: 500 mL; column length: 700 mm; column diameter: 30 mm; eluent flow rate: 12 mL / hr; fraction volume: 8.0 mL; number of fractions: 95; sample volume: 5 mL; separation temperature: 4 °C; elution buffer: 20 mM Tris-HCl, 1 mM NaN_3; pH 8.0. The peripheral tools used for preparative native GPC are listed in [24, 36]. The denoted molecular weights of the detected metal compounds are approximated values. Metal cofactors eluted in the range of the void volume (120 to 140 mL) of this method were identified and quantified by ICP-MS or GF-AAS [24, 36].

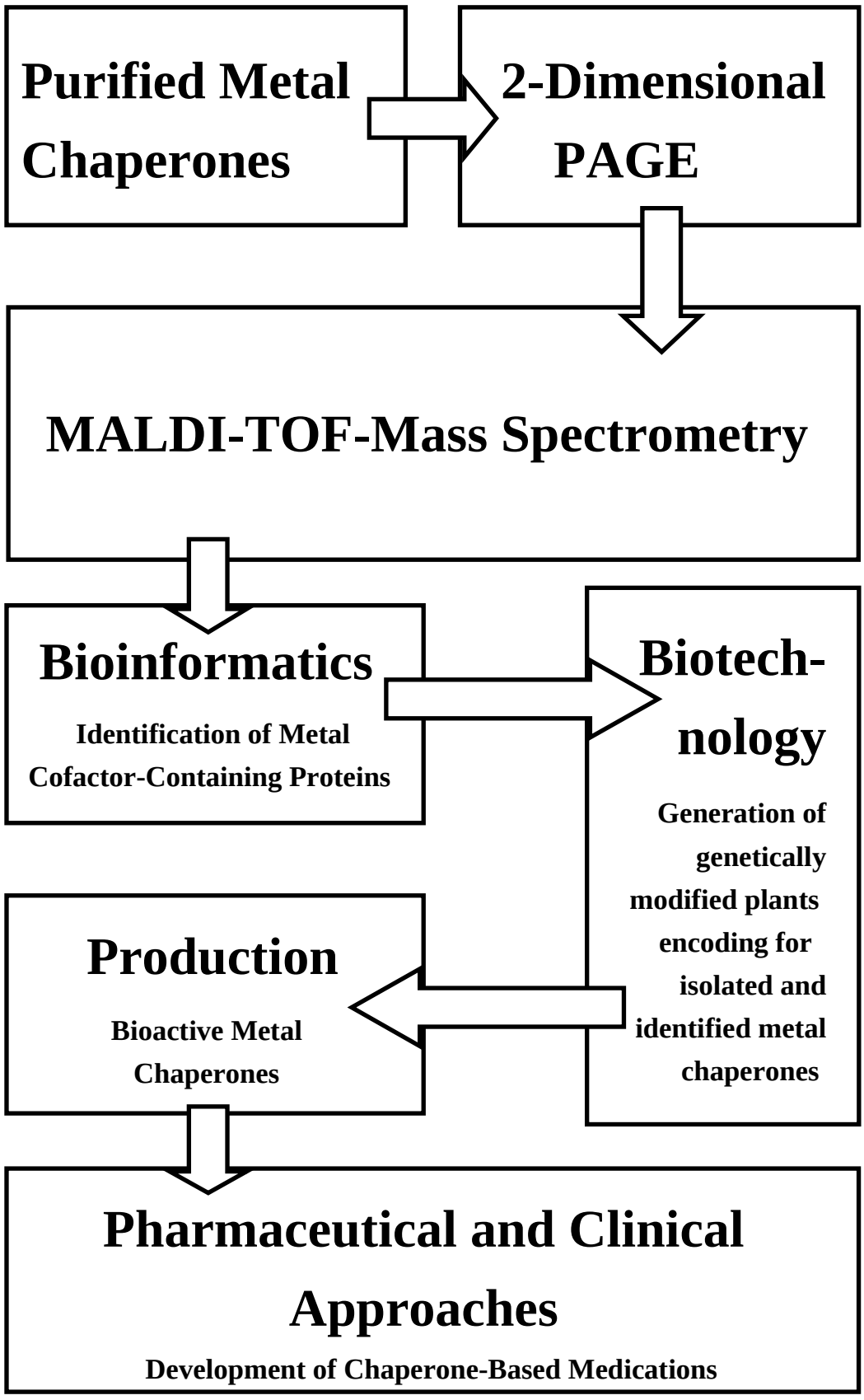

Fig. (3). Workflow schemes in metalloproteomics and phytofarming.