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Proteomics analysis of the actions of grape seed extract in rat brain: Technological and biological implications for the study of the actions of psychoactive compounds

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Abstract

Grape seed extract (GSE) is a commonly available dietary supplement taken for the anti-oxidant activity that's attributed to its proanthocyanidin (oligomers of monomeric polyphenols) content. Similar polyphenol-enriched preparations from blueberries and soy have shown protection against ovariectomy-induced or age-related cognitive deficits, suggesting that the molecular changes induced by these polyphenol preparations correlated with behavioral benefit. We hypothesized that ingestion of polyphenol-enriched preparations such as GSE would be manifested as protein changes that would be consistent with neuroprotection. Proteomics technology, namely 2D gel electrophoresis and mass spectrometry, identified quantitative changes in specific proteins induced in adult rat brain following ingestion of a powdered preparation of GSE. As recently reported [Deshane, J., Chaves, L., Sarikonda, K.V., Isbell, S., Wilson, L., Kirk, M., Grubbs, C., Barnes, S., Meleth, S. and Kim, H., 2004. Proteomics analysis of rat brain protein modulations by grape seed extract. Journal of Agricultural and Food Chemistry 52, 7872-7883.], the direction of change for the majority of the affected proteins was opposite to the direction the proteins were changed in either Alzheimer disease or in transgenic mouse models of dementia. A conservative conclusion is that GSE has neuroprotective activity, by affecting specific proteins in particular ways. In this chapter, elements of proteomics-type analysis are discussed that demonstrate the power of the technology to enable discovery of proteins involved in the response of the brain to a stimulus whether it be a dietary supplement, or a psychoactive drug. The fact that GSE affects proteins implicated in cognitive disorders suggests moreover that GSE may have impact on the actions of psychoactive drugs by maintaining an overall viability of the nervous system.

Keywords: Grape polyphenols; 2D gels; Botanicals; Grape seed; Psychoactive drugs

Introduction

More and more evidence points to the validity of the expression, "we are what we eat". Those who start each day with a refreshing cup of coffee depend on the positive effects of that dietary element for brain function. These are *psychoactive effects*, a descriptor usually reserved for chemicals with generally agreed upon negative connotations. As with all other organs, the viability and health of the brain are affected by genetic as well as by environmental factors. The latter modulate the system at both the protein and gene level, and include oxidative stress, as well as ingested chemicals from foods and dietary supplements. The purpose of this chapter is to present recent data regarding actions of a dietary

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supplement in mammalian brain obtained with proteomics technology, and to share with researchers how this technology allows us to "discover" whole repertoires of protein changes in complex tissues like the brain that occur following exposure to chemicals whether they be in the form of food ingredients, dietary supplements or psychoactive drugs. Ultimately, we want to know how molecular information of this sort can be used to determine the pathways involved in brain dysfunction or addiction. These proteins can be identified and quantified using proteomics technology, which enables analysis without the investigator knowing a priori which proteins might be involved.

Chemicals in foods and in dietary supplements that have effects on neural cell viability and/or function have the potential to modulate the actions of psychoactive drugs, either positively or negatively. The effects of the dietary supplements may occur directly in the brain, or may result from alteration of the pharmacokinetics of the psychoactive drug. It is therefore important to understand the molecular basis of the health benefits of dietary supplements taken for brain health. As part of the NIH-funded Purdue/UAB Botanicals Center for Age-related Disease, our laboratory has focused on the potential health benefits of grape-derived dietary supplements, specifically grape seed extract (GSE) preparations, in the mammalian brain. Epidemiological data have correlated lowered estrogen levels after menopause with higher incidence of various chronic conditions, including osteoporosis, heart disease and cognitive impairment (van der Mooren and Kenemans, 2004). In view of their structural similarities with 17β-estradiol, and with each other (Fig. 1), the polyphenols enriched in soy, green tea and grape skins have been studied intensively in recent years for potential estrogen-like actions (Vaya and Tamir, 2004), as well as for anti-oxidant activities (Cos et al., 2004). In this chapter, we summarize a proteomics analysis carried out on brain homogenates from normal rats that were given a diet supplemented with a high but nontoxic level of grape seed extract, enriched in proanthocyanidins, oligomers of the catechins. A systematic image analysis of the 2D gel dataset generated from the study coupled with rigorous statistical analysis enabled identification of protein gel spots that were reproducibly quantitatively different between the brains of rats given control diets versus GSE-supplemented diets. Mass spectrometry analysis then identified the proteins using established methods of peptide mass fingerprinting and LC-tandem mass spectrometry. The direction of the changes compared with those published for several of the proteins in studies of diseased brain by others, and the functional categories represented by the affected proteins, were consistent with the hypothesis that ingestion of GSE is neuroprotective. The experimental approach that generated these findings, as well as the findings themselves, are discussed in relation to the study of the actions of psychoactive compounds in the brain.

Experimental procedures

The procedures below were described in detail recently (Deshane et al., 2004).

Animals

35-day old Sprague–Dawley female rats were purchased, and maintained on a casein-protein based rodent diet (AIN-76A) for 1 week, after which they were segregated into 2 dietary groups, one which received AIN-76A supplemented with 5% GSE, and one which received AIN-76A only. Five animals were in each group. The animals were maintained in these 2 diets for 6 weeks, after which they were sacrificed by asphyxiation with carbon dioxide, and the whole brains dissected out above the brain stem. The brains were snap frozen in liquid nitrogen, and then stored at −80 °C until analyzed.

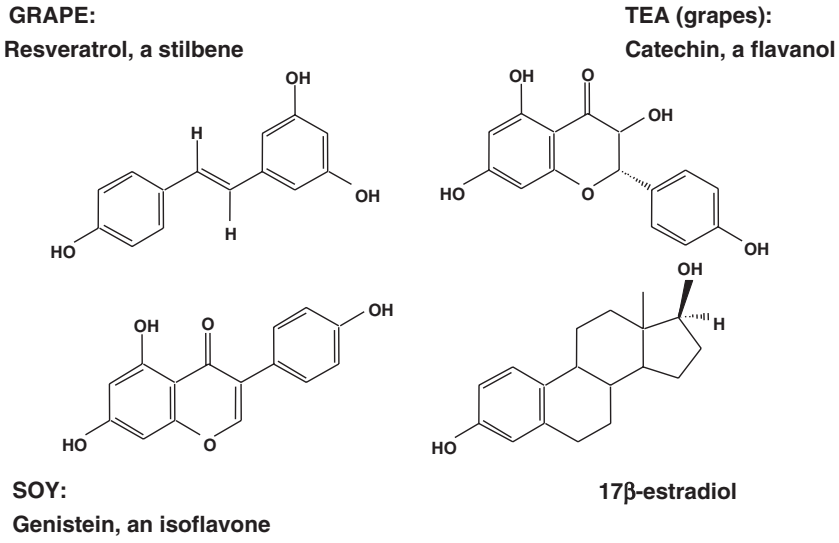

Fig. 1. Structural relationships between plant polyphenols and mammalian 17β-estradiol. Resveratrol, genistein and catechin represent the different classes of polyphenols in foods and supplements that are thought to have health benefits upon ingestion, due either to their similarity with estrogens (represented by 17β-estradiol), or to their capacity as anti-oxidants. Though resveratrol is commonly studied as representing grape polyphenols, grape skins and seeds are in fact enriched in proanthocyanidins, oligomers of catechins, thus the parenthetical reference to grapes next to TEA.

2D electrophoresis

Whole rat brains from the dietary groups were pulverized under liquid nitrogen, then homogenized in standard isoelectric focusing (IEF) buffer containing urea, thiourea, CHAPS, and tributylphosphine, adjusted with Complete protease inhibitor (Roche Diagnostics, Indianapolis, IN). After clarification by centrifugation, the supernatant was assayed for protein content, and an aliquot diluted to 200 μg protein per 200 μl with IEF rehydration buffer (IEF buffer containing ampholytes and a trace of Bromophenol blue). For each sample an immobilized pH gradient (IPG) strip containing a linear pH 4–7 gradient was rehydrated overnight over the 200 μl of sample, after which the IPG strips were electrofocused following manufacturer's instructions on a IPGphor IEF unit (GE Healthcare, Piscataway, NJ). Following IEF, the strips were stored overnight at −80 °C, then equilibrated into SDS-PAGE sample buffer prior to being laid across the top of the 2nd dimension SDS-PAGE gel and subsequent electrophoresis. To visualize the protein spots, the gels were stained with colloidal Coomassie Brilliant Blue G according to manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). Triplicate 2D gels were run for each biological sample; thus a total of 30 2D gels were generated. Fig. 2 shows how the animal study was carried out and how the samples were processed through the 2D electrophoresis procedure.

Image analysis and statistical analysis

The images of the Coomassie Blue-stained gels were acquired with a Bio-Rad GS 710-Calibrated Imaging Densitometer, and image analysis was performed via PDQuest software (Bio-Rad Laboratories, Hercules, CA); in brief, a digitized Gaussian master gel was generated from the gel with the most spots, and all other gel spots in the dataset were "detected" against this master gel. The spot intensities and the x, y coordinates were exported out of PDQuest and subjected to statistical analysis using Statistical Analysis Systems (SAS V. 09, SAS Institute, Cary, NC). Two sample t-tests and F-tests identified gel spots that differed significantly either in intensity, or in the variability of the intensity, respectively. These spots were then analyzed by Stepwise Discriminant Analysis (Mardia et al., 1995) to identify spots that discriminated 100% between the two groups.

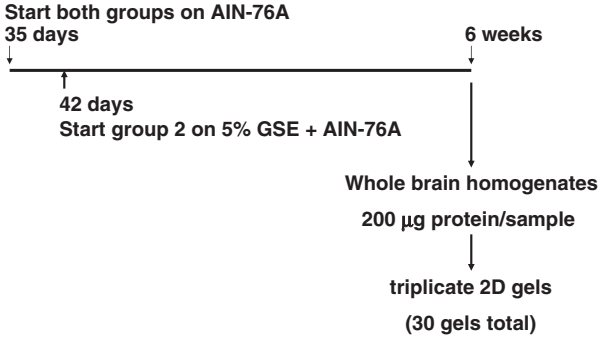

Fig. 2. Generation of brain homogenates for analysis of the effects of GSE in rat brain by 2D gel proteomics. 35 day female Sprague–Dawley rats were started on AIN-76A on day 35. One group of 5 was then switched to 5% GSE supplemented AIN-76A on day 42, while the other stayed on AIN-76A. After 6 weeks, all animals were sacrificed, and whole brains above the brain stem were dissected out and homogenized and processed for 2D gel analysis as described in Experimental procedures. A total of 30 2D gel images comprised the dataset from this experiment.

Protein identification

Peptide mass fingerprint analysis

The statistically significant gel spots were excised, and subjected to in-gel digestion with trypsin according to established procedures (http://donatello.ucsf.edu/ingel.html). After overnight digestion and extraction into 50% acetonitrile/5% formic acid, the peptides were diluted 1 : 10 with a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co, Milwaukee, WI) dissolved in acetonitrile : 0.1% TFA (1 : 1), spotted onto a MALDI-TOF target plate, and subjected to peptide mass fingerprint (PMF) analysis with a Voyager De-Pro mass spectrometer (Applied Biosystems, Inc., Foster City, CA) (Shevchenko et al., 1996). Samples were analyzed in the positive mode with delayed extraction. The acceleration voltage was set at 20 kV and 100 laser shots were summed. The instrument was externally calibrated with bradykinin, angiotensin, and neurotensin, and internal calibration was accomplished using the autolysis products of trypsin. In most cases, samples were processed on a robotic autosampler under the control of PS1-software (Applied Biosystems, Inc.). The raw MS data were de-isotoped and a peak list generated for submission to the MASCOT (http://www.matrixscience.com/) search engine in order to query the nonredundant NCBI database to potentially identify the protein. The parameters used for searching allowed for the inclusion of all species, one missed cleavage site and peptide mass tolerance set to 100 ppm. In some cases where the automatic analysis yielded ambiguity, the MS spectra were processed manually, also using MASCOT.

LC-tandem mass spectrometry

To confirm polypeptide matches generated by the PMF analysis, tryptic digests were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a hybrid quadrupole orthogonal time-of-flight mass spectrometer (Qtof2, Waters, Manchester, UK). Tryptic peptides were resolved by liquid chromatography (LC) using a FAMOS micro autosampler switching device (Switchos) and Ultimate nanoflow LC (LC Packings, San Francisco, CA). An aliquot of each sample was chromatographed on a 75 μm i.d. × 15 cm column packed with Biowide C_{18} 3 μm 300 A° (Supelco) in 0–80% acetonitrile gradient in 0.1% formic acid at a flow rate of 200 nl/min and the eluate passed into the nanoelectrospray interface of the Qtof2 mass spectrometer. Multiply charged molecular ions were subjected to collision-induced dissociation (CID) with argon gas and the resulting MS/MS data were recorded using automated MS to MS/MS data-dependent scanning, switching at a threshold of 6 counts.

Results

Image analysis of effects of oral intake of GSE on rat brain proteins

For the rat brain samples in this study, it was determined empirically, based on the number of spots detected and overall spatial resolution of the spots, that a linear 4–7 pH gradient in the first dimension and an 8–16% acrylamide gradient in the 2nd dimension gave optimal 2-dimensional resolution at the 200 μg loading (Fig. 3). The latter protein load, while somewhat overloaded for the major cytoskeletal proteins, enabled detection and analysis of the remaining observable proteins. It should be noted that the high-speed centrifugation of the homogenates prior to rehydrating the IPG strips was essential to deplete the samples of microparticulate matter, as well as lipids that resulted in anomalies in the 2nd dimension gels, including unacceptable streaking. Image analysis via PDQuest indicated that among the 30 gels in the dataset (triplicates of each of 10 biological samples), there were 124 spots common to the GSE gels, 127 common to the control gels. Of these sets of common spots, 90 were in common among all 30 gels.

Statistical analysis

Only spots that appeared on at least 2 of 3 replicate gels were included in the statistical analysis. Statistical analysis confirmed that several of the gel spots indicated by the image analysis were quantitatively different between the gels representing the 2 dietary groups (Fig. 3). Spots were different between the 2 groups either in intensity, or there was a difference in the number of spots for that protein in the horizontal dimension (evidence of isoform complexity or post-translational modifications). For certain proteins, the reproducibility of the isoform complexity in the control gel, and the shift to a different pattern, in the gel representing the GSE brain, was remarkable (Fig. 4), attesting to the reproducibility of the commercially prepared gels (both dimensions), as well as the overall technology.

Statistical analysis of the exported image analysis data (spot x, y coordinates, spot intensities) revealed a number of gel spots that were significantly different in intensity or in variability of intensity (1); stepwise discriminant analysis revealed that of the spots that differed significantly in intensity, only 7 were required to discriminate 100% between the 2 groups of gels. These 7, along with an additional 5 that were determined empirically to contain sufficient protein for mass spectrometry analysis, were excised from the gels, and processed by MALDI-TOF MS and LC-MS/MS to determine the identities of the proteins.

Specificity of the proteins affected by GSE

The validity of the described study was the demonstration that GSE, a complex mixture of complex chemicals, affected specific proteins in the brains of animals that ingested GSE, in a statistically significant manner. These proteins are shown in Fig. 5, in a modified Venn diagram that shows the relationship of these findings to those published recently by others. The proteins affected by GSE included those involved in energy generation (creatine kinase, brain isoform [CKB]), protein folding (heat shock protein 60 [hsp60], heat shock cognate protein 70 and 71 [hsc 70 and hsc 71]), and cytoskeletal proteins (neurofilament protein light chain [NFL-L] and medium chain [NFL-M], and glial fibrillary acidic protein [GFAP]). It is noteworthy that while the list was not long, the majority of those determined to be significantly affected by GSE were affected in a direction opposite to that detected for the same protein in either Alzheimer disease (Schonberger et al., 2001; Tsuji et al., 2002) or in transgenic mouse models of dementia (Tilleman et al., 2002a,b). These relationships are depicted in Fig. 5; specifically NFL-L and α-enolase were shown to be differentially expressed in the opposite direction to that in GSE-exposed brains in all three brain tissues, AD and the two mouse models of dementia, CKB, γ-enolase, and hsp60 were shown to be differentially affected in two studies in the opposite direction to that measured in this study by GSE, and five, GFAP, 14-3-3ε, hsc70 and hsc71, and vimentin, were shown to be affected

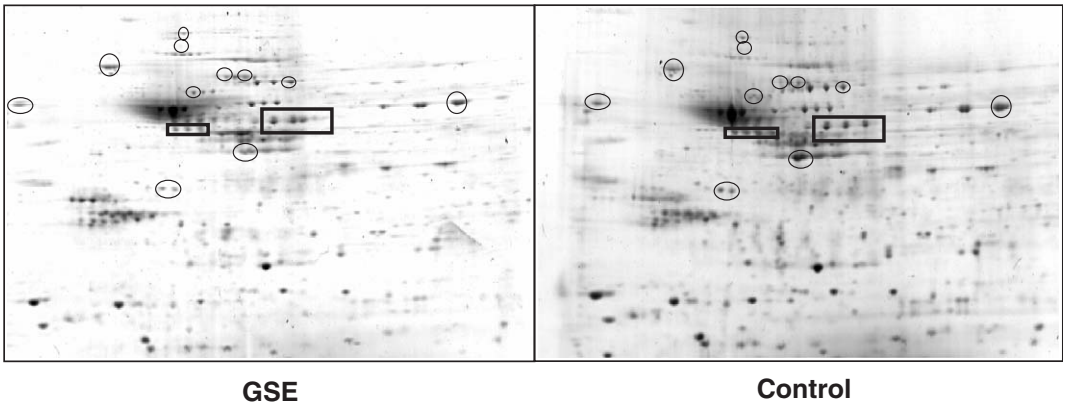

Fig. 3. Representative pair of 2D gels showing the differences in protein expression and modifications between GSE and control dietary groups. The circled spots were different in intensity; the boxed spots were different in isoform complexity.

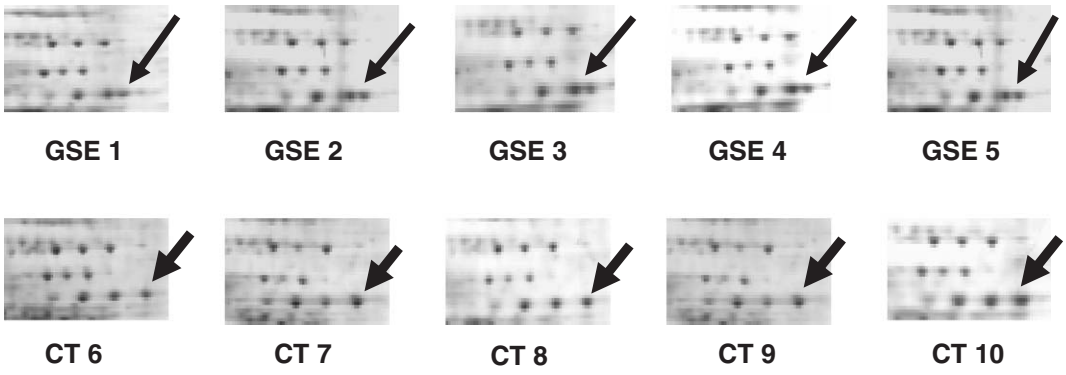

Fig. 4. Consistent difference in isoform complexity detected between GSE and control gels for selected proteins. Of the set of 3 spots spaced equidistant from each other in the control (CT) gels, the most basic(right-most) spot was consistently shifted to a more acidic position in the GSE gels. All spots in the horizontal "string" of 3 spots in both cases were identified as CK-BB by peptide mass fingerprinting and LC-MS/MS as described in Experimental procedures. GSE 1–5 and CT 6–10 represent the 5 animals in each dietary group.

in at least one other study (see Deshane et al., 2004, Table 1 for full details).

Discussion and concluding remarks

The data presented here emphasize the power of the proteomics technology to resolve reproducible patterns of proteins in complex biological samples such as brain. To accomplish this, the experimental design and analytical conditions must be carefully examined and controlled. Especially with organs such as brain, where different regions of tissue represent different functions, investigators must decide whether to examine the entire brain or a specific region, keeping in mind that analyzing the proteome of the entire brain or even of a specific region may result in important loss of information. In the present study, the pH range 4–7 was chosen based on empirical observation that this gave the best overall spatial resolution of the brain proteins focused. However, if a particular group of proteins are of interest in the experiment, e.g., the cytochrome *P*450s, several of which have p*I* values > pH 7, a different pH range may be more appropriate. Finally, since protein abundance may range over 6–9 orders of magnitude, a decision has to be made as to whether to prefractionate the proteome using subcellular fractionation, biochemical or immunological methods. Remarkably, statistically significant differences were detected for several proteins in this study without incorporating a subfractionation step; however, ongoing experiments are addressing the likely possibility that the detected protein differences are enhanced in specific brain regions.

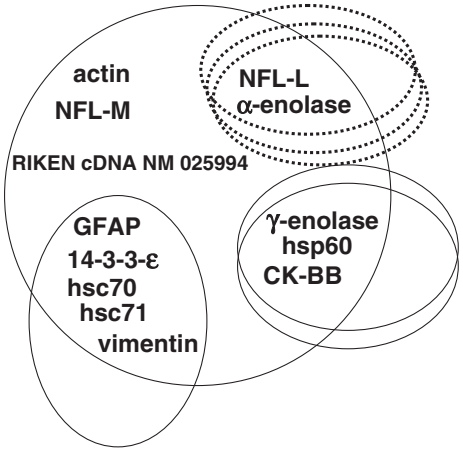

Fig. 5. Modified Venn diagram, showing the overlap among proteins determined to be different in the brains of animals exposed to GSE, and in either AD brain, or transgenic models of neurodegeneration. Of the 13 proteins determined to be significantly different in GSE versus control brains, 2, indicated by the triple dotted line, were shown to be differentially affected in all tissues representing dementia studied thus far, 3, indicated by the double circles, were shown to be differentially affected in 2 studies, and 5 proteins indicated by the single overlapping circle were shown to be differentially affected in at least one other study. Not included in any overlapping circles were actin, NFL-M and RIKEN cDNA NM 025594, which this study has identified for the first time as candidate proteins implicated in brain health.

It is worth pointing out that in no case did the statistical analysis indicate a protein was altered more than 2-fold between the GSE and the control gels. A pilot study with the same preparation of GSE showed that the latter protected against ovariectomy-induced cognitive impairment in rats (Peng et al., 2003). The combination of the behavioral data and the proteomics and statistical data described here suggest that distinct physiological effects of food or dietary supplement components can result from small changes in amounts of proteins. It should be kept in mind that this study examined one time point. It will be important to examine other time points after administering the GSE, to determine if other protein changes occurred earlier, with perhaps greater magnitude of change. It is not unreasonable to consider that a system in attempting to maintain homeostasis even in response to "beneficial" stimuli such as GSE, might "dampen" large initial changes in protein amounts, so that small changes would ultimately be detected for these proteins at later time points.

As stated in Results, the majority of the effects on the proteins detected in this study could be described as "protective" against age- or pathology-related cognitive impairment for the reasons depicted in Fig. 5. As such, it will be interesting in future experiments, to examine brain regions known to be involved in the cognitive functions of learning and memory, such as the hippocampus and frontal cortex. If the protein changes detected in this study are truly protective, the

prediction is that the same proteins will be affected in the hippocampus and frontal cortex, but with greater magnitude of change, relative to those measured in whole homogenate.

The experiment described here was the first to utilize a global approach to identify specific proteins affected in a target tissue by a complex dietary supplement taken orally. Previous studies have identified or studied specific proteins affected by either pure polyphenols or extracts of polyphenol-enriched dietary supplements in disease models (Hibasami et al., 2004; Lamartiniere et al., 2002; Bagchi et al., 2002, Yamakoshi et al., 1999; Smith and Luo, 2003). This is the first study however to identify specific proteins, utilizing a global approach, nonselective for any particular proteins. The challenge will now be to assess which of these is actually contributory to a neuroprotective effect, and which are statistically "real" but a benign consequence of ingestion of GSE.

The principal goal of this chapter was to share the different aspects of 2D gel proteomics technology with those interested in determining the molecular basis of the actions of psychoactive compounds. An added value from the presented proteomics study is the idea that ingestion of a dietary supplement such as GSE might in fact mitigate or accentuate the actions of some psychoactive drugs, if the overall effect of the GSE is neuroprotective. In other words, if the ultimate effect of GSE is an overall enhancement of neural tissue viability, could this have subtle but real effects in attenuating potentially toxic actions of psychoactive drugs. Alternatively, could ingesting something like GSE affect whether a particular individual will become addicted to particular psychoactive drugs. Questions such as these can be addressed using the same GSE preparation, and the same proteomics technologies to determine the complement of proteins affected by psychoactive drugs of interest. These can be divided into those that are altered by prior intake of GSE, or by intake simultaneous with the drug. While these are speculative statements, much is at stake, since addiction to psychoactive compounds continues to be a major public health issue that affects both adults as well as the youth.

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