Biochemical Comparison of Commercial Selenium Yeast Preparations

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Abstract The trace mineral selenium (Se) is an essential element for human and animal nutrition. The addition of Se to the diet through dietary supplements or fortified food/feed is increasingly common owing to the often sub-optimal content of standard diets of many countries. Se supplements commercially available include the inorganic mineral salts such as sodium selenite or selenate, and organic forms such as Se-enriched yeast. Today, Se yeast is produced by several manufacturers and has become the most widely used source of Se for human supplementation and is also widely employed in animal nutrition where approval in all species has been granted by regulatory bodies such as the European Food Safety Authority (EFSA). Characterisation and comparison of Se-enriched yeast products has traditionally been made by quantifying total selenomethionine (SeMet) content. A disadvantage of this approach, however, is that it does not consider the effects of Se deposition on subsequent digestive availability. In this study, an assessment was made of the water-soluble extracts of commercially available Se-enriched yeast samples for free, peptide-bound and total water-soluble SeMet. Using LC-MS/MS, a total of 62 Se-containing proteins were identified across four Se yeast products, displaying quantitative/qualitative changes in abundance relative to the certified reference material, SELM-1 (P value <0.05; fold change ≥2). Overall, the study indicates that significant differences exist between Se yeast products in terms of SeMet content, Se-containing protein abundance and associated metabolic pathways.

Introduction

Selenium (Se) is an essential micronutrient for humans and animals playing important roles in antioxidant function, thyroid hormone metabolism, reproduction and immune responses [1]. Supplementation of farm animal diets with Se is stimulated by the beneficial effect of this element on animal health and, consequently, by the improved quality of foodstuffs of animal origin [2]. Furthermore, several studies on animal models, cell lines and human intervention trials have demonstrated cancer protective effects of selenium [3].

The compounds available for use as Se supplements include the inorganic forms, sodium selenite and sodium selenate, and the organic forms, L-selenomethionine (SeMet) and Se-enriched yeast [4]. Although the metabolism of both organic and inorganic Se forms shows certain similarities, not all of these forms are metabolised alike, and humans have been found to absorb and retain Se better from SeMet than from the inorganic Se salts [5–7]. In a number of studies in humans and animals, in particular, those on Se-deficient diets, the bioavailability of Se from Se-enriched yeasts and the bioavailability of SeMet has been shown to be approximately 1.5- to 2-fold higher than that of inorganic forms of Se [8, 9]. Consequently, the biological actions of Se depend not only on the amount but also on the form of the Se source [10] and on the digestibility and accessibility of selenoamino acids from the protein-rich yeast matrix [11].

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Proteins containing Se in the form of SeMet residues are not formally classified as selenoproteins but rather called selenium-containing proteins [12]. Therefore, in the case of organic Se products such as Se yeast, biological efficacy is more dependent on the digestibility and accessibility of Se-containing proteins and peptides present in commercial preparations. An assessment of the composition of Se yeast products in the marketplace indicated that significant differences exist between the products [13]; however, speciation studies traditionally focused on the determination of low molecular mass selenocompounds [12].

In recent years, the methodology used in classical proteomics has been gradually incorporated into analytical speciation studies. The application of both elemental and organic mass spectrometry methods, usually preceded by liquid chromatography, has become commonplace in studies on selenoproteins and selenium-containing proteins [14]. Proteomic techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) enable the study of the vast nature of proteins and the correlation with their underlying biological processes. This allows the identification of their abundance changes, levels of production, post-translation modification, amino acid substitutions and polymorphisms to be determined. For this reason, physiological and nutritional alterations can play a pivotal role in altering the proteome of an organism facilitating the differentiation and discrimination of Se yeast products [15].

In this study, an assessment was made of the water-soluble extracts of Se-enriched yeast samples for free, peptide-bound and total water-soluble SeMet. LC-MS/MS was used to identify and quantify individual proteins present in the soluble extracts. Protein identities were validated using UniProt, and Gene Ontology classification was performed to define the potential biological role of the identified proteins and their relevance to animal health [16]. Characterisation of Se yeast in this way provides a unique fingerprint of origin and accounts for fate of Se during the production process, which is paramount importance for regulatory and safety agencies [17]. The Se-enriched yeast certified reference material (SELM-1) was used for the calibration of instruments and evaluation of methods for the determination of SeMet, Met and total Se in yeast. This appears to be the first reported study using gel-free and label-free quantification for the proteomic assessment of water-soluble extracts of Se-enriched yeast products.

Materials and Methods

Selenised Yeast Samples

Samples from three non-consecutive production batches of four commercially available Se yeast products were used in all biochemical and proteomic analyses. The certified reference material, SELM-1, was the test material for the inductively coupled plasma mass spectrometry (ICP-MS) validation study and control for proteomic analyses [18].

Extraction of Free, Bound and Total Selenomethionine from Yeast Samples

Protease XIV and Trizma Base were obtained from Sigma. Methanol (HPLC grade) and trifluoroacetic acid (TFA) were supplied by Sigma. All chemicals used in this work were of analytical-reagent grade or higher unless otherwise specified.

Sample Extraction of Total SeMet

Approximately 0.04 g of Se yeast sample was accurately weighed into 1.5 mL microcentrifuge tubes. Protease enzyme solution (13.33 mg Protease XIV in 0.5 mL of Tris buffer pH 7.5) was added and vortex mixed for 2 min. Samples were ultrasonicated for 25 s at 80 % amplitude and the probe washed with 250 μL enzyme solution into the 1.5 mL tube. Some ice and water were placed into the microwave carousel, and the microcentrifuge tubes were placed into the carousel holder (up to 14 tubes per run). The extraction program was operated for 15 min at a power output of 30 W. Extracted samples were centrifuged at 14,000 rpm for 3 min. Supernatants were then transferred in to 15 mL centrifuge tubes. Pellets were washed with water (0.9 mL) and vortex-mixed until completely in solution. Samples were centrifuged at 14,000 rpm for 6 min and supernatants added to the 15 mL centrifuge tubes. This step was repeated three times. Volumes were adjusted to 15 mL using water and mixed well before removing an aliquot (2 mL) for filtration (0.25 μm) and dilution before analysis.

Sample Extraction of Water-Soluble Free SeMet

Approximately 0.5 g of Se yeast sample was accurately weighed into 50 mL sterilin tubes. HPLC-grade water (5 mL) was added. Samples were placed on a shaker for 15 min at 300 rpm. Samples were then centrifuged at 8500 rpm for 10 min. Aliquots (2 mL) of supernatants were filtered (0.25 μm) and diluted before analysis.

Sample Extraction of Water-Soluble-Bound SeMet

Water extracts (500 μL) were added to 13.33 mg of protease. Samples were ultrasonicated for 25 s at 80 % amplitude and the probe washed with 250 μL enzyme solution (6.667 mg Protease XIV/250 μL) into 1.5 mL tubes. Some ice and water were placed into the microwave carousel, and the microcentrifuge tubes were placed into the carousel holder (up to 14 tubes per run). The extraction program was operated for 15 min at a power output of 30 W. The extracted samples

were centrifuged at 14,000 rpm for 3 min. Supernatants were then transferred into 15 mL centrifuge tubes. Volumes were adjusted to 15 mL using water and mixed well before removing an aliquot (2 mL) for filtration (0.25 μm) and dilution before analysis.

Instrumentation and Calibration

Separations were performed on an Agilent Zorbax RX-C8 4.6 × 250 mm 5 μm column using an Agilent Technologies 1260 infinity series LC system connected to an Agilent Technologies 7700× series ICP-MS. The mobile phase was water/methanol/TFA (97.9:2.0:0.1) at a flow rate of 1 mL/min, and the column temperature was set at 30 °C.

A Heinemann 130W ultrasonic-homogeniser (HTU SONI 130, USA) equipped with a 3 mm double step titanium probe was used for sample preparation. A CEM Discover microwave equipped with an Explorer SP-D Plus 24/48 autosampler was used for sample extraction. An ELGA Purelab Flex S7 HPLC water system was used to produce >18 MΩ cm water, and samples were filtered using Chromafil Xtra RC-20/25. The HPLC mobile phase was filtered with Whatman Glass Microfibre Filters.

SeMet standard powder was obtained from Sigma (>98 % by TLC). This was used to prepare a 100 mg/L stock solution in 0.1 M HCl. Aliquots (0.1 mL) of this solution were frozen and used fresh each day.

The instrument was calibrated over the range of 50 to 250 ppb SeMet (20.15 to 100.75 ppb Se as SeMet) as described previously [19].

Sample Analysis for ICP-MS

Aliquots (2 mL) of the sample extracts were filtered into 2 mL microcentrifuge tubes. Further dilutions were made using HPLC-grade water and transferred to 2 mL HPLC vials for analysis. Each sample was analysed in triplicate using an injection volume of 45 μL and monitored for isotopes ^{76}Se, ^{77}Se and ^{78}Se.

Sample Preparation for LC-MS

Protein concentration of water extracts (1/10 dilution) was quantified using the Bradford assay, and all solutions were adjusted to the same concentration. Approximately 150 μg of protein was precipitated from each sample using TCA/acetone precipitation. Samples were solubilised in 6 M urea/2 M thiourea solution, and all samples were again adjusted to the same concentration as necessary. Samples were reduced and alkylated, with DTT and IAA, respectively, followed by overnight trypsin digestion in the presence of ProteaseMax (Promega) mass spectrometry-compatible detergent [20]. Digestion activity was quenched by acidification,

and samples were desalted using C_{18} ZipTips (Millipore) prior to analysis. Desalted samples were dried to completion and resuspended in loading solvent (2 % (v/v) acetonitrile, 0.05 % (v/v) TFA) immediately prior to LC-MS/MS analysis.

LC-MS/MS Analysis

The analysis of the water extracts was performed using a reverse phase column (15 cm × 75 μm inner diameter) packed with 3 μm C_{18} particles (EASY-Spray; Thermo Scientific). All samples were separated on a 2 h acetonitrile gradient (5–35 %) in 0.1 % formic acid at a flow rate of 300 mL/min, using the UltiMate 3000 Nano LC system from Dionex. The LC was coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) with up to the top 15 most abundant isotope patterns selected for fragmentation (MS/MS) (Top 15 method). The Q-Exactive was operated in data-dependent mode with survey scans (MS) acquired at a resolution of 70,000 at m/z 400, while MS/MS scans were acquired at a resolution of 17,500.

Data Analysis

The MS data were analysed using MaxQuant version 1.3.0.5 [21] with protein identification achieved through comparison against the *Saccharomyces cerevisiae* reference strain S288C database (www.yeastgenome.org). Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines, acetylation of N-terminals and selenium-sulphur substitution were set as variable modifications. The maximum peptide/protein false discovery rates (FDR) were set to 1 % based on comparison to a reverse database. The label-free quantitative (LFQ) algorithm was used to generate normalised spectral intensities and infer relative protein abundance. Proteins that matched to a contaminants database or the reverse database were removed, and proteins were only retained in final analysis if detected in at least two samples from at least one product. Quantitative analysis was performed using a *t* test to compare pairs of samples, and proteins with significant change in abundance (*P* value <0.05; fold change ≥2) were included in the quantitative results. Qualitative analysis was also performed, to detect proteins that were found in at least two batches of a particular product, but undetectable in the comparison product.

Results and Discussion

Determination of Total, Free and Bound Selenomethionine

There is a common misconception that the level of SeMet is an appropriate indication for the bioavailability of selenised yeast preparations [22]. In actuality, selenium bioavailability

depends on the digestion of Se-containing yeast, the absorption of Se from the intestinal tract and finally the transport and biotransformation of Se into biologically active forms [11]. Therefore, estimation of adequate levels of Se in the diet requires information not only of the total amount of Se but also of the bio-accessible species in the sample [6, 23, 24]. Se-enriched yeast has been extensively studied and fractionated [12, 25] with SeMet reported as the major selenoamino acid in water-soluble yeast extracts, replacing Met by simple competition. Se yeast products consist of dried and inactivated preparations of *S. cerevisiae*; the final product is a light to dark brown, free-flowing powder readily dispersible in water and organic solvents [26–28]. Aqueous extraction allows the recovery of 15–25 % of Se [12] present predominately as protein-bound SeMet [29, 30]; therefore, an enzymatically assisted extraction method is typically used to determine total SeMet content [11]. Essentially, larger SeMet-containing proteins and peptides are broken down into free amino acids and short-chain peptides liberating the selenoamino acid for detection [19]. Figure 1 shows the difference between the five yeast products used in this study in terms of free, peptide-bound and total water-soluble selenomethionine ($P < 0.01$).

There was significant variation noted in the product specific inter-batch levels of free, peptide-bound and total water-soluble SeMet ($P < 0.01$) except for yeast product 2, which did not display such inconsistency ($P = 0.1$ and 0.012, respectively). Product 1 had the highest variability in peptide-bound water-soluble SeMet across the three batches assessed. This may be due to the age of the sample or alterations that may have been made to the fermentation process between batches [31]. Another factor which may contribute to variation in final concentration of SeMet between batches and/or products is the number of washes applied post-production. Typically, the residual sodium selenite used during production is

removed by washing the yeast cream prior to drying [32]. It has been proposed that some SeMet in yeast may not be incorporated chemically into Se-containing proteins but physically associated with cell wall constituents [3, 23]. As commercial Se yeast preparations consist of dried and inactivated fragments of *S. cerevisiae*, it is reasonable to assume that some leaching may occur post-production and prior to drying [12].

Product 4 consistently contained the most free SeMet in each batch, whereas product 3 contained the least. It is important to make this distinction between total SeMet and free SeMet, as only the free form of this selenoamino acid is available for non-specific incorporation into proteins in place of methionine, leading to Se-containing proteins, representing a biological pool of Se [7]. Unless selenium-containing proteins and peptides are digested to liberate SeMet as a free amino acid, then regardless of total Se and/or total SeMet content, the Se source must undergo a metabolic transformation to selenide prior to its assimilation [24, 33]. Hinojosa-Reyes et al. [11, 34] reported that 89 % of total Se was extracted after in vitro gastrointestinal (GI) digestion of a selenised yeast standard, but surprisingly, only 41 % was quantified in the form of free SeMet. Therefore, although this free selenoamino acid appears to be the most abundant Se species in GI digests, half of the total SeMet content in the extracts still seems to be associated with Se-containing peptides after in vitro GI digestion [35]. A similar study by Encinar et al. [12] examined the composition of three commercial preparations of Se-enriched yeast in terms of Se deposition within individual yeast fractions. Each product was extracted with water followed by a variety of enzymic digestions intended to liberate selenocompounds associated with various polysaccharide and protein portions. Their study on the recovery of the different classes of Se species demonstrated significant differences between Se yeast samples from different sources.

Fig. 1 Selenomethionine partitioning in commercially available yeast products. [a,b,c,d,e]Average values that lack a common superscript letter differ in significance ($P \leq 0.05$) ($n = 9$)
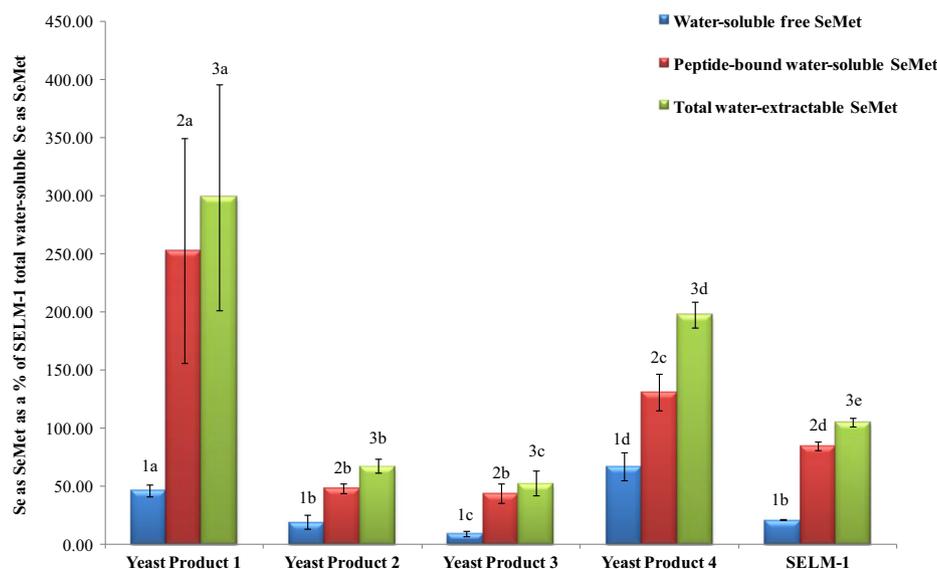
Similarly, based on the values reported in the present study, it is apparent that the profiles of water-extractable SeMet differ markedly, indicating that all sources of 'selenium yeast' are not alike. It is clear that the compartmentalisation of Se within yeast matrix is different between preparations and is strain-dependent [36] and, consequently, it is reasonable to expect that these preparations will also differ in parameters such as shelf-life, bioavailability and, indeed, toxicology [37]. How these data related to actual bioavailability of selenium within the animal can only be speculative [13]; therefore, simulated in vitro GI digestion studies will be required to establish a complete overview of bioavailable intake [11, 38].

Although enzymatic hydrolysis is a powerful tool for distinguishing between fractions, it is clear that there are inherent difficulties with enzymatic release of SeMet which may lead to lower recoveries [39]. For instance, SeMet may not be fully liberated from the protein, peptide or other cellular components with which it is associated, and it may be solubilised in forms which get lost during the derivatisation or chromatography steps or may interact non-specifically with sample components [40]. It must also be noted that Se-containing proteins are labile and prone to degradation after being extracted [41] leading to an underestimation of the Se content. However, inclusion of the certified reference material, SELM-1, in intercomparison Se speciation and quantitation studies, validates the reported values [18].

A top-down proteomic approach incorporating liquid chromatography with tandem mass spectrometry can also alleviate issues with extraction, enzymatic digestion and incomplete derivatisation [42]. In addition, a deeper insight into the identity of the proteins can be obtained by tryptic digestion and investigation of the Se-containing peptides formed [43].

### Determination of Inter-batch and Inter-product Protein Content Variation Between Se Yeast Products

In addition to the determination of the level of organic Se (as SeMet), a series of analytical methods are routinely used to ensure the quality and origin of the finished Se yeast product. These include assessment of strain purity using biochemical and genetic identification techniques, measurement of moisture control and quantification of toxic impurities such as lead, arsenic and cadmium [39]. In recent years, proteomic techniques such as LC-MS are increasingly used to measure changes in protein abundance across the samples that are being compared [44]. Relative quantifications allow the accurate determination of protein ratios in samples that are genetically or physiologically similar or diverse [45]. To determine the variation in protein content between batches and products, label-free mass-spectrometry-based proteomic analysis, scatter correlation plots and hierarchical clustering were performed. As previously mentioned, a proteomic approach such as LC-MS/MS can circumvent potential issues with extraction, enzymatic digestion and incomplete derivatisation associated with Se speciation studies [40].

Scatter plots of protein intensities revealed high correlation (low variation) between batches of the same products, indicating good reproducibility of batches and technical preparation of samples (Fig. 2a–e). Pearson correlation coefficients (where 1 = identical) were calculated between all pairs of batches within a product group, and these indicated that the lowest variability was observed within the SELM-1 product group (Pearson coefficient 0.983–0.985) which is to be expected as there is only one production batch of this certified reference material (CRM) available commercially. As the SELM-1 water extract was prepared in triplicate from the same batch, the low variation in this sample is reflective of the high reproducibility of the sample analysis, i.e. protein extraction, digestion and LC-MS analysis. All batches clustered to their individual product groups except for one. Yeast product 2 sample C clustered closer to SELM-1 than the other products, which may have resulted from an overall high similarity between yeast product 2 and SELM-1 (Fig. 3: Pearson coefficient 0.948). Overall, these results indicate high sample reproducibility, which is of paramount importance for label-free quantitative (LFQ) proteomic analysis [46].

For determination of inter-product variation, a comparison of all five products was also carried out using scatter plot analysis and hierarchical clustering. The normalised intensities of all proteins were generated by MaxQuant analysis, these values were $\log_2(x)$ transformed and the mean value was calculated for each product group. Yeast products 2–4 and SELM-1 all demonstrated a high level of correlation (Fig. 3: Pearson coefficient >0.84 for all pairs) which makes these products suitable for analysis within an LFQ proteomic experiment [47]. However, LFQ proteomic analysis revealed that yeast product 1 clustered independently of all other products based on protein composition (Fig. 3; Pearson coefficient <0.56 for all pairs). Hierarchical clustering again confirmed this poor correlation, with yeast product 1 grouping away from the other Se-enriched products (Fig. 4a) thus highlighting the need for multiple statistical analyses. The poor correlation between yeast product 1 and all other products is in agreement with the inconsistencies noted for SeMet partitioning in this present study and previous studies addressing the composition of Se yeast products from different sources [12]. This extent of variation makes yeast product 1 unsuitable for comparison in an LFQ proteomic experiment, which is normally predicated on the basis that the majority of proteins remain unchanged in a comparison set [48]. With yeast product 1 excluded from the analyses, a total of 786 proteins were identified across the remaining four products, of which 374 proteins displayed quantitative/qualitative changes in abundance relative to SELM-1 (P value <0.05; fold change ≥2) (Supplementary Table 1).

Fig. 2 Multi-scatter correlation plots of four selenium-enriched products and SELM-1 reference material. Scatter plots demonstrating the variation within product batches for yeast products 1–4 (**a**–**d**), SELM-1 (**e**); batches/replicates tested are indicated (*A–C*). Axis values represent the $\log_2(x)$ value of the normalised intensity of each protein. *Values in blue* are Pearson correlation coefficients and indicate the level of variation between sample groups (colour figure online)
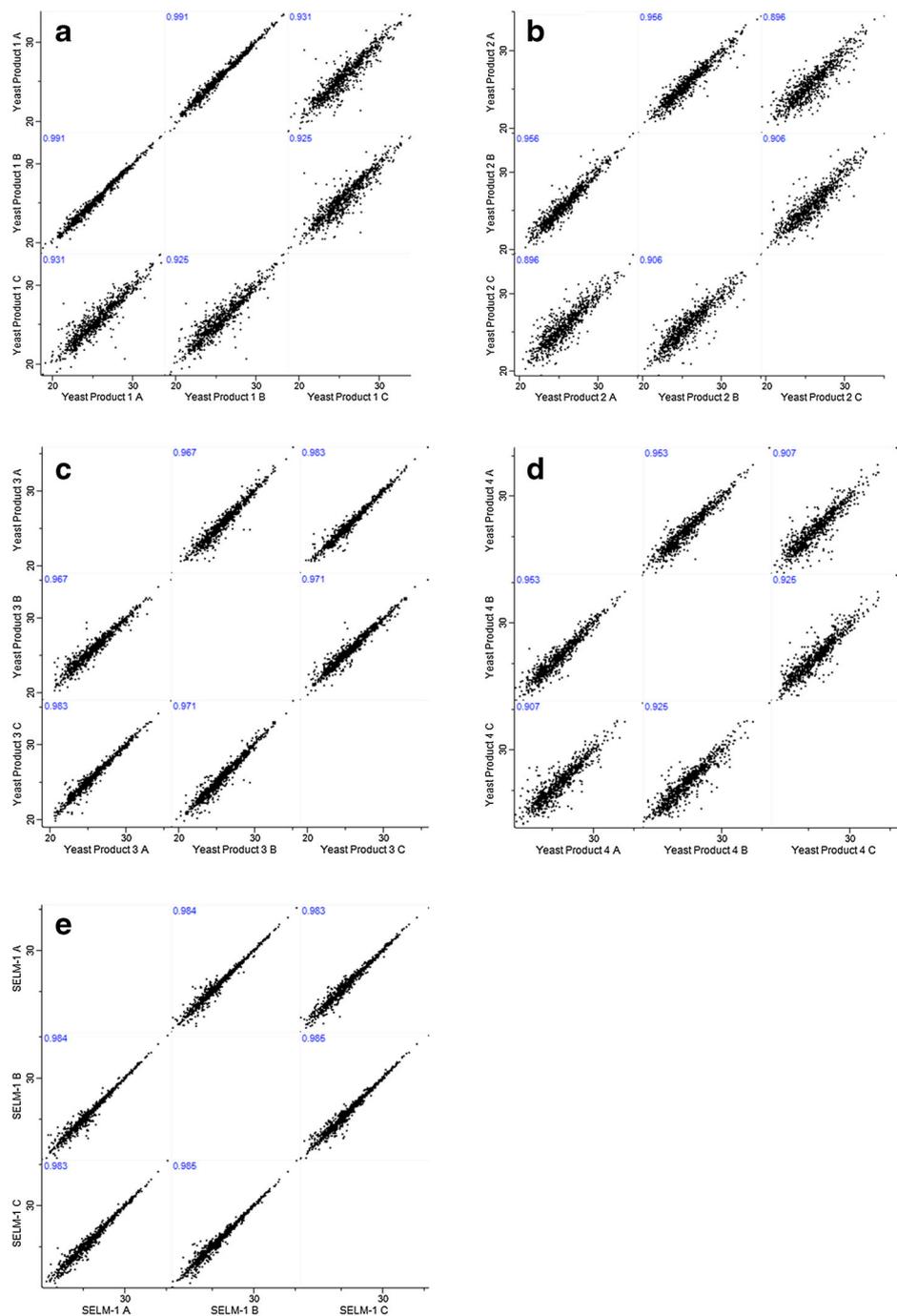
Product 2 demonstrated the least disparity from SELM-1, and these products were also seen to cluster together on the heat maps (Fig. 4) again indicating high strain or product similarity. Although proteomic analysis revealed overall batch reproducibility of individual products, it also successfully differentiated outliers and highlighted potential inconsistencies in production processes, which is in agreement with the previously discussed SeMet standard deviation. In addition, although SeMet partitioning indicated similar concentrations for products 2 and 3 in terms of peptide-bound water-soluble SeMet, hierarchical clustering revealed that product 3 is in fact the most dissimilar to product 2 and, indeed, all other products. A deeper insight into protein abundances and identities (Fig. 4) can be achieved by further interrogation of the data for Se-containing proteins.

Fig. 3 Multi-scatter correlation plots of four selenium-enriched products and SELM-1 reference material. Scatter plots demonstrating the variation within product batches for yeast products 1–4 and SELM-1. Axis values represent the $\log_2(x)$ value of the normalised intensity of each protein. Variation between the products, based on the mean log-transformed intensity for each protein, is shown. *Values in blue* are Pearson correlation coefficients and indicate the level of variation between sample groups. Yeast product 1 (*red box*) demonstrated poor correlation to all of the other products analysed (Pearson coefficients <0.56) (colour figure online)
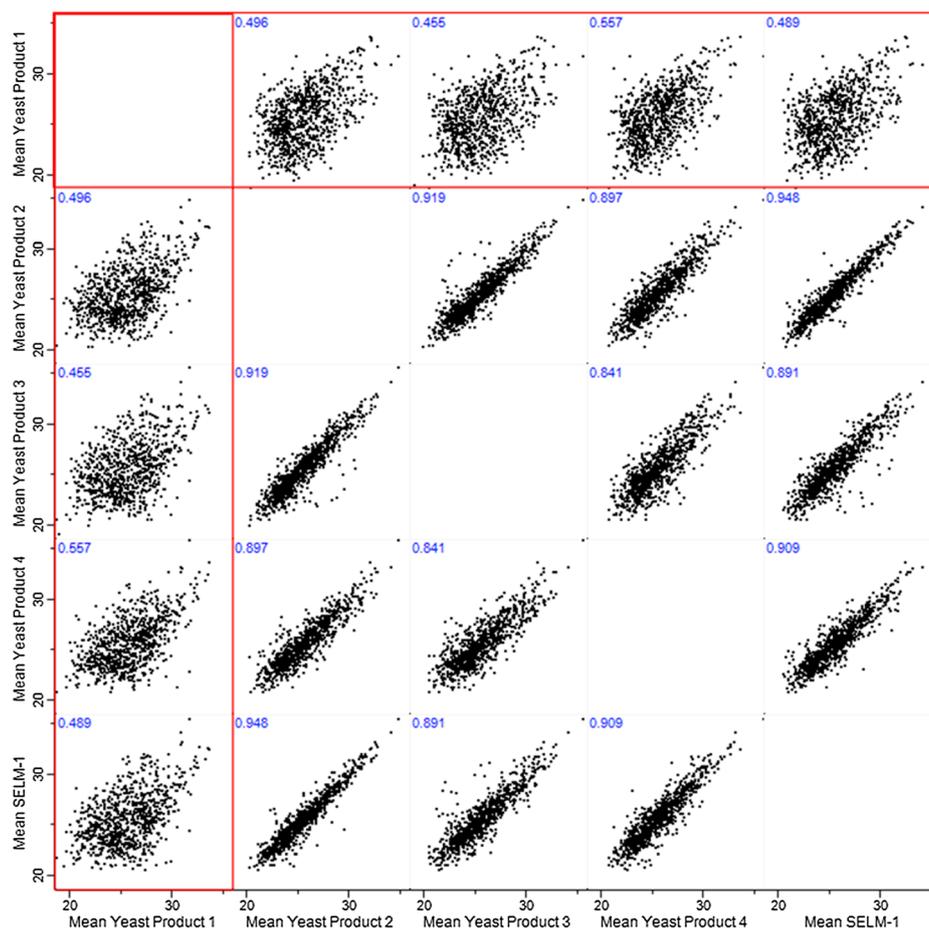
Analysis for Se-Containing Proteins

Interrogation of the data generated from the four products (excluding Yeast product 1) revealed the identification of a number of selenopeptides (n = 186 unique peptides), corresponding to 116 unique protein groups. These modifications were associated with 172 SeMet replacements and 14 selenocysteines (SeCys). Proteins identified with SeMet/SeCys containing peptides were extracted from the quantitative results (Supplementary Table 1), and this subset is presented in Table 1 (62 proteins in total). A description of each protein is provided along with its respective fold-change in abundance (fold change ≥2) relative to SELM-1.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) has regularly expressed its view that all SeMet sources would result in similar selenium deposition (EFSA 2011a, b, 2012); this view was later extended to the hydroxy-analogue of SeMet (HMSeBA) (EFSA, 2013). Since L-SeMet is the predominant selenocompound incorporated into proteins of Se yeast, the panel concluded that there was no reason to assume that free L-SeMet would result in an essentially different deposition pattern [49]. However, as SeMet is incorporated randomly into proteins as a substitute for the sulphur analogue Met [25, 37, 50], the

degree of expression of particular proteins depends on the yeast strain and the fermentation process [17] as clearly evidenced in Table 1.

The abundance changes reported are irrespective of Se modification and represent the overall abundance of that protein in each of the products compared to SELM-1. The proteins presented in Table 1 are a subset of the overall quantitative results which have been selected based on the fact that selenium modifications were also detected in these proteins. For example, the relative levels of alcohol dehydrogenase (Adh1) are measured based on all identified peptides (modified and unmodified). SeMet containing peptides were detected in Adh1 from products 2, 3 and SELM-1, but the overall abundance of the Adh1 protein was significantly higher in product 3 compared to SELM-1. Proteins with Se modifications including Ahp1, Bmh1, Eno1, Eno2, Sip18 and Mmf1 were also identified in previous studies on Se yeast [16, 25, 50, 51] thus corroborating the results presented in Table 1. Three different isoforms of glyceraldehyde-3-phosphate dehydrogenase (Tdh1, Tdh2 and Tdh3) were also identified. GAPDH is ubiquitous in yeast and has previously been reported to incorporate SeMet randomly within the protein [17]. In general, yeast supports a high degree of SeMet incorporation, by replacing about 30 % of all Met with SeMet [24, 25, 50].
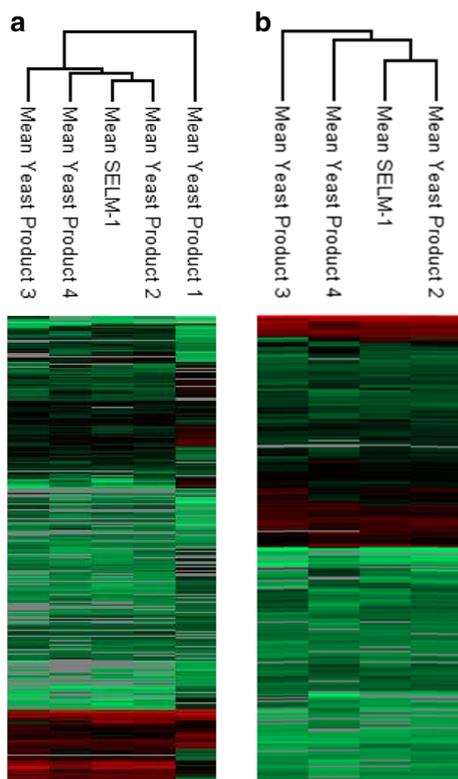
Fig 4 Heat maps demonstrating the hierarchical clustering of **a** yeast products 1–4 plus SELM-1 and **b** yeast products 2–4 plus SELM-1 (product 1 excluded), based on relative protein abundances. Low-abundance proteins are depicted in *green* and high abundant proteins are depicted in *red*. *Grey shading* indicates that protein was not identified in the relevant product (colour figure online)

However, within peptides from the same protein, SeMet/Met ratios will vary depending on the differential exposure to oxidative or enzymatic degradation of SeMet side chain and its position in the protein (buried or exposed), and also to the change in the production rate of each protein after Se addition to growth media [50]. Differences in protein content might also be ascribed to diverse manufacturing methods or bioprocessing to which specific yeast is subjected during fermentation, recovery and drying operations [52–54]. For example, Tdh3 was identified in two products but at opposing abundance levels. As glyceraldehyde-3-phosphate dehydrogenase is involved in oxidation-reduction pathways, differential expression may be due to stress imposed by Se salt addition during fermentation, thus highlighting that a range of divergent manufacturing methods may used to generate Se-enriched yeast products [16, 39] resulting in inherent differences between preparations. It is clear that bioavailability of SeMet will be dependent not only on the abundance of a particular Se-containing protein but also on the position of SeMet residues within the protein; therefore, consideration must be given to the strain-specific deposition of Se into individual protein and peptide-containing fractions [55]. As Se yeast is produced commercially by methods which are

proprietary and/or patented [32, 56], there is a need for straightforward protocols like the one presented, allowing rapid characterisation and quantitation of Se-enriched yeast samples [57].

Beside SeMet, Se is known to occur in SeCys and is referred to as the 21st amino acid because its specific insertion into Se-proteins is directed by the SeCys insertion sequence (SECIS) element [17]. As the *S. cerevisiae* genome does not contain the SECIS element, SeCys in Se yeast can only be inserted by the competition of Se with sulphur in the Cys synthesis pathway, leading to the non-specific SeCys incorporation. SeCys was not formally identified in the selenoproteome of Se yeast until recently, when Bierla et al. demonstrated the considerable incorporation of SeCys in proteins of the yeast proteome despite the absence of the UGA codon [50]. Therefore, the identification of 14 SeCys modifications among four products in the present study significantly adds to recent discoveries. In essence, this proteome level characterisation of yeast products provides a unique fingerprint of product origin and reproducibility, which is essential for regulatory and safety agencies to approve Se-enriched yeast products for animal nutrition [17]. Further bioinformatic interrogation of the data using Gene Ontology analysis identified a number of biological processes that showed variation between each of the products compared to SELM-1.

Proteomic Comparison of Yeast Products to SELM-1 Using Gene Ontology Analysis

Proteomic analysis revealed 374 individual proteins that showed some change in abundance in either yeast product 2, 3 or 4 in comparison to the reference material SELM-1 (qualitative and quantitative results). In concordance with the previously reported hierarchical clustering heat maps, product 2 demonstrated the least variation from SELM-1, with 26 proteins showing decreased abundance and 27 proteins increasing in abundance compared to SELM-1, whereas product 3 demonstrated the most variation from SELM-1, with 112 proteins showing decreased abundance and 171 increasing in abundance. Bioinformatic interrogation of the data using Gene Ontology analysis revealed a number of biological processes that showed variation between each of the products compared to SELM-1. A number of biological processes were altered/affected in the individual products (when compared to SELM-1), including translation, transcription, transport and oxidation-reduction processes. The net number of proteins that were higher or lower in abundance in each product, categorised by biological process and relative to SELM-1, is shown in Fig. 5.

It is well recognised that Se plays a key role in several biological processes, including the response to oxidative stress, DNA damage and repair [4, 58]. The results presented in Fig. 5 demonstrate differential protein abundance between

Table 1 SeMet/SeCys-containing proteins with quantitative/qualitative changes in abundance relative to SELM-1

Fold Change Yeast Product 2 v SELM-1	Fold Change Yeast Product 3 v SELM-1	Fold Change Yeast Product 4 v SELM-1	Gene Name	Protein IDs	Peptides	Sequence coverage [%]	Mol. weight [kDa]	Protein Description
	-2.6954		ADH1	YOL086C	20	61.8	36.849	Alcohol dehydrogenase
		4.8329	ADH2	YMR303C	14	37.1	36.731	Glucose-repressible alcohol dehydrogenase II
-2.9690	-3.7902	-2.4416	AHP1	YLR109W	12	89.2	19.114	Thiol-specific peroxiredoxin
	2.0078		ALD4	YOR374W	22	58.6	56.723	Mitochondrial aldehyde dehydrogenase
	Unique to SELM-1		BMH1	YER177W	14	50.6	30.091	14-3-3 protein, major isoform
	-3.7915		CMD1	YBR109C	13	97.3	16.135	Calmodulin
		-2.1744	COX4	YGL187C	13	79.4	17.142	Subunit IV of cytochrome c oxidase
		-5.3034	CYC1	YJR048W	12	67	12.182	Cytochrome c, isoform 1
		-4.4841	DDR48	YMR173W	16	65.3	46.233	DNA damage-responsive protein
	2.2927		EGD2	YHR193C	7	47.7	18.709	Alpha subunit of the heteromeric nascent polypeptide-associated complex (NAC)
	-2.1225		ENO1	YGR254W	31	81.2	46.816	Enolase I
	-2.1858		ENO2	YHR174W	33	84.7	46.914	Enolase II
		-5.4184	GAS5	YOL030W	9	25	51.869	1,3-beta-glucanosyltransferase
-2.0275			GIS2	YNL255C	8	60.1	17.103	Translational activator for mRNAs
		2.7239	GND1	YHR183W	15	39.9	53.543	6-phosphogluconate dehydrogenase
	-43.6679	-3.4679	HMF1	YER057C	9	96.9	13.906	Member of the p14.5 protein family with similarity to Mmf1p
	-3.8409		HNT1	YDL125C	4	27.2	17.679	Adenosine 5'-monophosphoramidase
	2.6416		HSP10	YOR020C	7	71.7	11.372	Mitochondrial matrix co-chaperonin that inhibits the ATPase activity of Hsp60p
		Unique to Product 4	HSP82	YPL240C	26	37	81.405	Hsp90 chaperone required for pheromone signaling and negative regulation of Hsf1p
		-3.0302	HYP2	YEL034W	10	75.2	17.114	Translation elongation factor eIF-5A
	3.9702		IGD1	YFR017C	10	64.6	21.783	Cytoplasmic protein that inhibits Gdb1p
-3.5665	-6.7146		LEU2	YCL018W	25	77.5	38.952	Beta-isopropylmalate dehydrogenase (IMDH)
	-3.2827		MET6	YER091C	42	59.5	85.859	Cobalamin-independent methionine synthase
		-2.6696	MLC1	YGL106W	16	81.9	16.444	Essential light chain for Myo1p, light chain for Myo2p
	-28.6555	-19.3447	MMF1	YIL051C	6	58.6	15.908	Mitochondrial protein
		2.6396	MSC1	YML128C	16	35.9	59.588	Protein of unknown function
	2.3653		NHP6A	YPR052C	8	63.4	10.802	High-mobility group (HMG) protein
	2.0664		NHP6B	YBR089C-A	7	53.5	11.476	High-mobility group (HMG) protein
	-3.2049	-2.1213	OYE2	YHR179W	7	20	45.01	Conserved NADPH oxidoreductase containing flavin mononucleotide (FMN)
	5.8820	-3.6778	PCK1	YKR097W	12	23	60.983	Phosphoenolpyruvate carboxykinase
	-2.4620	2.6759	PET10	YKR046C	17	59.7	31.246	Protein of unknown function that co-purifies with lipid particles
-2.0013	-2.7838		PGM2	YMR105C	28	61.5	63.088	Phosphoglucomutase
-3.5104	-3.2205		PNC1	YGL037C	4	28.7	24.993	Nicotinamidase
	4.4443		PRB1	YEL060C	13	21.1	69.621	Vacuolar proteinase B (yscB)

Fold changes are based on protein intensities as calculated using MaxQuant LFQ algorithm and only proteins with a significant change in abundance ($P <$ 0.05; $\geq$2-fold change in abundance) are included. Positive numbers indicate increased abundance and negative figures indicated decreased abundance, relative to the levels in SELM-1. Qualitative differences are also included and indicated as unique to a specific product. Values in red represent proteins with a higher abundance in products 2, 3 or 4 relative to SELM-1, and green indicates a lower abundance of that protein relative to SELM-1 (quantitative or qualitative) (colour table online)

Table 1   (continued)

Fold Change Yeast Product 2 v SELM-1	Fold Change Yeast Product 3 v SELM-1	Fold Change Yeast Product 4 v SELM-1	Gene Name	Protein IDs	Peptides	Sequence coverage [%]	Mol. weight [kDa]	Protein Description
	2.0046		QCR6	YFR033C	4	32	17.257	Subunit 6 of the ubiquinol cytochrome-c reductase complex
	2.3357		RDL1	YOR285W	10	89.9	15.413	Protein of unknown function containing a rhodanese-like domain
	2.1302	-2.5972	RNQ1	YCL028W	6	21.7	42.579	[PIN(+)] prion
	3.1941		RPL31A	YDL075W	6	55.8	12.953	Protein component of the large (60S) ribosomal subunit
	-2.1036		RPN10	YHR200W	3	12.3	29.747	Non-ATPase base subunit of the 19S regulatory particle (RP) of the 26S proteasome
	2.2727		RPS21B	YJL136C	8	70.1	9.7597	Protein component of the small (40S) ribosomal subunit
	2.0833		RPS28B	YLR264W	2	31.3	7.5647	Protein component of the small (40S) ribosomal subunit
	-3.2101		RTN1	YDR233C	15	52.2	32.916	ER membrane protein
-3.0527		-4.6430	SIP18	YMR175W	4	40.5	8.8738	Phospholipid-binding hydrophilin
	2.0810	-2.2447	SMT3	YDR510W	4	46.5	11.597	Ubiquitin-like protein of the SUMO family
	-33.7974		SOD2	YHR008C	8	58.8	25.774	Mitochondrial manganese superoxide dismutase
	3.8648		SPG4	YMR107W	13	85.2	13.18	Protein required for survival at high temperature during stationary phase
	-2.9821		TDH1	YJL052W	24	70.8	35.75	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1
	-3.7794		TDH2	YJR009C	18	63	35.846	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2
	-2.6172	2.5432	TDH3	YGR192C	26	84.3	35.746	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3
		2.1526	TEF2	YBR118W	15	40	50.032	Translational elongation factor EF-1 alpha
		5.7402	TFS1	YLR178C	10	65.3	24.357	Protein that interacts with and inhibits carboxypeptidase Y and Ira2p
	4.0509		TMA17	YDL110C	8	61.3	16.771	Protein of unknown function that associates with ribosomes
		-2.3043	TPM2	YIL138C	19	75.8	19.093	Minor isoform of tropomyosin,
		-5.6927	TRX1	YLR043C	7	66	11.235	Cytoplasmic thioredoxin isoenzyme
	2.3791	3.9860	UIP4	YPL186C	7	25.3	34.221	Protein that interacts with Ulp1p
		-5.7427	VHS2	YIL135C	8	23.4	47.96	Cytoplasmic protein of unknown function
	Unique to SELM-1		YDL218W	YDL218W	3	18.6	34.449	Putative protein of unknown function
	-2.4504		YGR169C-A	YGR169C-A	3	37	10.495	Putative protein of unknown function
		-2.6021	YIL002W-A	YIL002W-A	3	53.6	7.7286	Putative protein of unknown function
		-3.0034	YMR074C	YMR074C	10	63.4	15.977	Protein with homology to human PDCD5
	6.8073	2.5860	YNL195C	YNL195C	15	85.8	28.321	Putative protein of unknown function
	-3.2666		YOR020W-A	YOR020W-A	3	35.6	9.6176	Putative protein of unknown function

Se-enriched yeast products in a number of biological processes. Thus, the routes by which each of the individual product compounds are metabolised and the relative abundance of their metabolites will be entwined with the efficacy of Se compounds in disease prevention and treatment [31]. It is also worth noting that the water extraction used in this study is vastly different from volatile conditions encountered in the GI tract where conditions will solubilise some proteins and
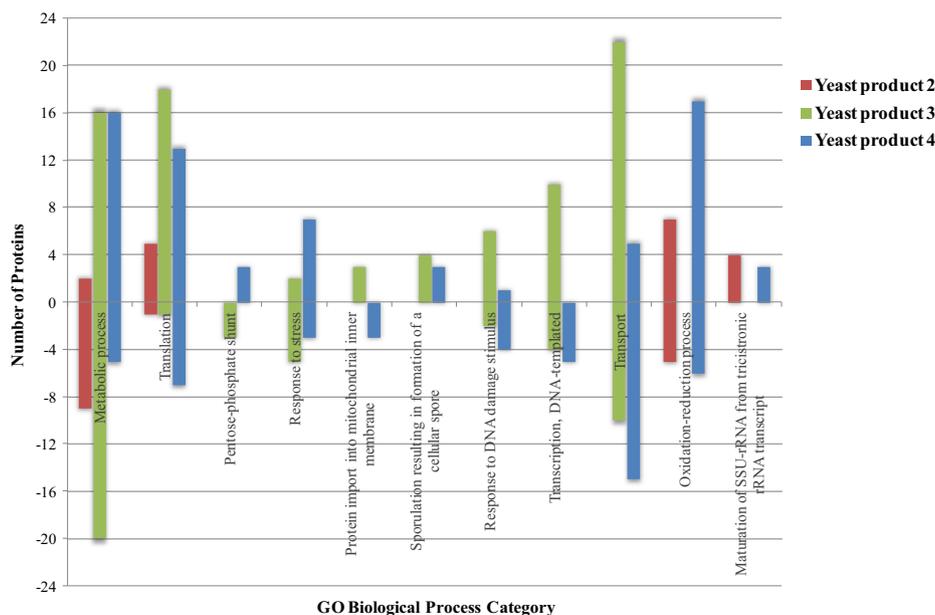
destabilise others [59]. Therefore, the extent to which the active forms of yeast proteins identified can be transported intact across the intestinal barrier, and the biological effect of such proteins or corresponding peptides must be determined in pre-clinical animal models before any definitive conclusions can be made on potential beneficial health effects [16]. Nevertheless, knowledge of a group of proteins that respond to Se supplementation will be useful in the assessment of a particular metabolic status [60].

KEGG Metabolic Pathway Analysis

Using KEGG pathway analysis [61] and FungiFun analysis [62], the enzymes with altered abundances relative to SELM-1 were mapped on the glutathione metabolism and Cys/Met metabolism pathways (Fig. 6). These pathways were previously observed to be altered in response to Se, and Se-containing proteins that underwent a significant change in abundance have been identified (data not shown). Proteins involved in Cys/Met metabolism were mapped to show the effect of product difference on proteins from this pathway (Fig. 6a). For example, the protein encoded by *SPE3* (spermidine synthase), which utilises *S*-adenosylmethioninamine for the production of spermidine, demonstrated increased abundance in product 4 only, once again demonstrating differences between the products. This may signify an increase in the production of spermidine, which is associated with increased cell lifespan and suppression of oxidative stress in yeast [63].

One protein, Sam2, which is involved in Met conversion to *S*-adenosyl methionine (SAM) was observed at a significantly higher level in products 3 and 4 compared to SELM-1 (Fig. 6a). SAM, also known as AdoMet, is an essential

molecule that exists in all living organisms and is also a precursor molecule in the aminopropylation and transulfuration pathways [64]. It is distributed to virtually all body tissues and fluids and is of fundamental importance in a number of biochemical reactions involving enzymatic transmethylation, contributing to the synthesis, activation and/or metabolism of such compounds as hormones, neurotransmitters, nucleic acids, proteins and phospholipids [20, 65, 66]. SAM is effective in the treatment of clinical disorders such as liver disease, osteoarthritis and depression and has been used extensively as an approved prescription medicinal dietary supplement in Europe. Chu et al. [67] recently reported the urgent need to enhance the production of SAM to meet growing demand. Notably, *S. cerevisiae* has two distinct SAM-synthetase genes, named *SAM1* and *SAM2*, which arose from gene duplication [68] and share a high degree of similarity [69]. Although *SAM1* and *SAM2* encode functionally equivalent SAM synthetases, they are regulated differently [70]. Both genes undergo feedback repression by SAM, like other genes of sulphur aminoacid metabolism, but expression of *SAM2* also increases during growth, which overrides the SAM-mediated repression [64, 70]. Thomas et al. [69] also reported that *SAM1* and *SAM2* are regulated transcriptionally by excess methionine in the growth medium, but in an opposing manner. Expression of the *SAM2* gene is induced by the presence of methionine in the growth medium, whereas *SAM1* is repressed [69].

Interestingly, *S*-adenosylhomocysteine hydrolase (Sah1) and methionine synthase (Met6) are also involved in the SAM cycle, also known as the activated methyl cycle. Within this cycle, upon donating the methyl group, SAM is converted to *S*-adenosylhomocysteine (AdoHcy) which is then

hydrolyzed to homocysteine by Sah1 [71]. Inhibition of Sah1 normally results in an accumulation of cellular AdoHcy, which is often accompanied by a simultaneous rise in SAM because of the feedback inhibition by AdoHcy on most methylation reactions [72]. Therefore, as can be appreciated in Fig. 6a, the production of SAM is clearly influenced by choice of production strain and/or manipulation of culture conditions.

Similarly, a number of proteins associated with glutathione metabolism were detected (Fig. 6b). Proteins encoded by *OXP1*, *DUG1*, *GND1*, *GTT1*, *SPE3*, *RNR2* and *RNR4* were annotated to the glutathione metabolism KEGG pathway, which was significantly represented in the data (P = 0.00008; FungiFun analysis, data not shown). The chemical and physical resemblance between Se and S establishes that both these elements share common metabolic pathways [73]. In addition to being a constituent of cysteine and methionine, sulphur (S) is also present in glutathione, an essential metabolite in almost all eukaryotic organisms playing a key role in redox homeostasis and in the cellular response to oxidative stress [74]. Expression of glutathione *S*-transferase (Gtt1) is reportedly induced after diauxic shift and remains high throughout the stationary phase [75], while phosphogluconate dehydrogenase (Gnd1) is important for protecting yeast from oxidative stress [76]. Glutathione utilisation in *S. cerevisiae* has also been shown to be dependent on the presence of a glutathione transporter and an alternative pathway of

glutathione degradation that involves the 'Dug pathway complex' comprising Dug1 [74]. The remaining annotated proteins have peripheral roles in glutathione metabolism. For example, Oxp1 is a 5-oxoprolinase which functions to produce glutamate and normally forms part of the gamma-glutamyl cycle [77].

As *S. cerevisiae* is unable to distinguish between Se and S [73], it is unsurprising that Se supplementation affected the levels of these glutathione metabolism-related proteins in Se yeast products. Both Gtt1 and Gnd1 were observed to increase in abundance following Se enrichment and were present at significantly higher levels in product 4 compared to SELM-1. An additional glutathione transferase, Ecm4, was also uniquely identified in product 4. Other glutathione metabolism-related proteins were detected at significantly lower levels in either product 3 or product 2 relative to SELM-1. It is worth noting that SAM is also the principal biological methyl donor and a precursor for glutathione [78]. Once again, product 2 is identified to be the most dissimilar to all other products analysed; however, all products demonstrated differential protein abundance relative to SELM-1. Altogether, these results point to the pathways and systems that are affected following Se enrichment yet differ considerably among the Se-enriched yeast products examined.

Conclusion

To date, speciation data have made it possible to discriminate between yeast containing organic Se and yeast mixed with inorganic selenate or selenite, to distinguish Se-rich yeasts produced by different manufacturers and to verify batch-to-batch reproducibility. Recent advances in mass spectrometry and quantitative proteomics enable analysis of proteins in a much more comprehensive and high-throughput manner than previously possible. In addition, the availability of SELM-1 reference material has improved the quality assurance of the analyses of Se-enriched yeast. In this study, we have demonstrated that commercial Se-enriched yeast products differ significantly in terms of protein abundance and associated metabolic pathways in addition to SeMet content. In essence, all of the presented data confirm that Se yeast products are distinct from each other. This distinctness is due to the differential deposition of Se present within individual yeast fractions indicating that retention and thus bioavailability of Se from each of these products are different. Investigating the bioavailability of Se yeast products using in vitro digestion will further differentiate the products in terms of bio efficacy.
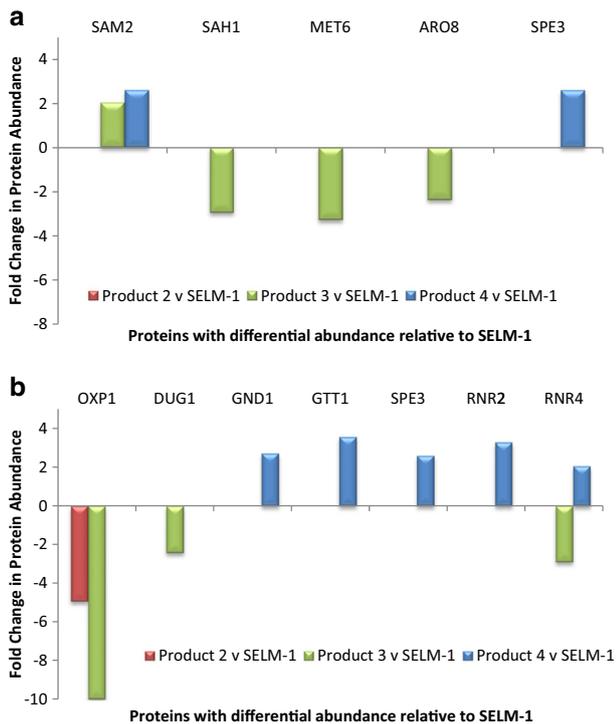

Fig. 6 a Cysteine/methionine-metabolism and **b** glutathione-metabolism related proteins with differential abundance relative to SELM-1. Gene names are indicated above the *x*-axis and fold difference of individual proteins is on the *y*-axis

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