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EFFECTS OF ALKALINITY ON EXTRACTION EFFICIENCY OF SELENIUM-CONTAINING PROTEINS FROM Se-ENRICHED YEAST

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ABSTRACT

The alkalinity of selenium-containing proteins extracted from Se-enriched yeasts was investigated in this work. Altogether four solvents, alcohol, KCl, KOH and deionized water were experimented for yeast protein extraction. With the Bradford method, it showed that 24.65% total proteins were extracted on the action of 0.1mol L^{-1} KOH. Another result showed that with the alkali method and with the concentration of 0.1mol L^{-1} KOH, a higher Se extraction yield of about 32% was produced. Also a speciation analysis was carried out. The speciation distribution revealed the protein would be denatured in an extremely high alkalinity condition. Therefore it was concluded that the alkali condition should be moderate and in this study it was seen that the optimal KOH concentration was 0.1-0.15 mol L^{-1}. The organic Se speciation was accounted for 11% of the total Se extracted and after the enzyme hydrolysis, the organic Se accounted for nearly 70% which was higher than other alkalinity. Furthermore, it was seen that most important Se species observed was SeMet-a combination with protein.

Keywords: Se-enriched yeast, selenium-containing proteins, alkali extraction method, alkalinity, Se speciation analysis

INTRODUCTION

Being a crucial component of glutathione peroxidase (GPx) and thioredoxin reductase (TrxR), selenium (Se) is an essential trace element because of its unique antioxidant properties and its ability to regulate thyroid gland metabolism. The toxicity, nutritional essentiality, and cancer preventive effects of Se are largely dependent on the Se species[1]. Selenium exists in plants and animal foods predominantly in organic forms such as selenoamino acids[2].

Most of the Se exists in Se-enriched yeast in the forms of selenium polysaccharide and selenium-containing proteins, which have a highly biological availability. The content of protein is as high as 40% and about 83% of the Se in the yeast was bound to yeast proteins[3]. Therefore, Se-enriched yeast is taken as a kind of nonpoisonous, natural and safe Se-supplement, and it is widely used nowadays. However, till now the enriched-yeast is directly used by human via solutions or addition agents, which showed a lower availability and efficiency compared with the extracted proteins.

The alkaline solutions have already been widely used as a practical and effective method for protein extraction from plant sources[4]. However, there is little study on the effect between alkali intensity and the selenium-containing proteins to choose the optimum alkali condition in which the protein can keep the best activity and integrity. In the present study, the relationship between the KOH concentration and the extract efficiency of total Se and its species will be discussed. Furthermore, the optimal KOH concentration to extract the total Se will be determined.

EXPERIMENTAL

Materials

In this study, commercially available yeast, Angle yeast (Angle Co. Ltd) was purchased. The concentration of total Se in the yeast powders was about 1850 mg g^{-1}. MilliQ water (Milford, USA) was used in the whole process and the following chemicals: Coomassie brilliant blue (CBB，solarbio), bovine serum Albumin (BSA, Sinopharm Chemical Reagent Co. Ltd)，Selenium standards including Se stock

standard solution (Sigma-Aldrich), Se-cystine ($SeCys_2$, Fluka Chemie, >98%), Se-methylselenocysteine (SeMeCys, Fluka Chemie, 98%) and Se-methionine (SeMet, Fluka Chemie, 99%) were involved. These stock solutions were kept at 4 ^oC. Protease XIV and Protease K (Sigma-Aldrich, 4 U mg^{-1}) were kept at -20 ^oC. All solvents/chemicals used were of the analytical grade.

Instrumentation

An ultraviolet spectrophotometry instrument (UV-9100，BFTP Co，Beijing) was used for determining protein content. The complementary techniques of HPLC-UV-HG-AFS were used to analysis the Se species. The HPLC system consisted of a Shimadzu LC20AB pump (Japan) and Hamilton PRP X100 columns (Hamilton, Reno, NV). All samples for Se detection were analyzed by the HG-AFS system (AFS-9230, Titan Co., Beijing).

Extraction

One aim of this work was to investigate the optimal methods for protein extraction from the Se-enriched yeast. The proteins, in general were classified into alcohol-soluble, water-soluble, alkali-soluble and salt-soluble categories. Thus, one could firstly use a certain acidity-buffer solution to extract low molecular mass species such as selenate, and selenite, amino acids and water-soluble selenoproteins; secondly with a 0.5 mol L^{-1} KCl solution to extract salt-soluble proteins; thirdly with a 75% ethanol to extract alcohol-solution proteins; and finally with a 0.1 mol L^{-1} KOH solution to extract alkali-soluble proteins[5].

In this study, deionized water, 0.5 mol L^{-1} KCl, 0.1 mol L^{-1} KOH and 70% ethanol were used respectively. Each 1 g sample and corresponding 30 mL solvent were added into a 50 mL centrifuge tube and the mixture was shaken for 24 h at 35 ℃. The extraction efficiencies were calculated according to the total Se concentration extracted from the Se-enriched yeast. The best solvent was then determined, based on the amount of total Se and the protein content extracted. Because of cell walls the yeast had, the tubes with samples were put into an ultrasound bath for fragmenting yeast cells for about 10 min, in order to release selenium more efficiently from cells and their components. The ultrasound parameters were listed below in Table 1.After extraction the mixtures were centrifuged for 30 min at 4000 rpm. Supernatants were separated from precipitations. The supernatants and the precipitations were stored at 4 ℃ until analysis.

Table-1: Parameters of the ultrasound process

Frequency (kHz)	Time(min)	Temperature(^0C)	Times
20	3	0	3

Determination of protein contents

The Bradford method is a good way for a quick determination of protein contents[6]. By measuring the standards of known concentrations (0, 20, 40, 60, 80, 100 μ g g^{-1}) of BSA protein solutions through the spectrophotometer, one can establish a calibration curve to determine the protein concentrations in unknown samples. The contents of proteins in all samples were determined as shown in Table 2.

Table-2: Protein contents extracted with different solvents determined by the Bradford method. Two replicated samples were used to provide an average.

Solvents	70% Ethanol		0.5mol L^{-1}KCl		Deionized Water		0.1mol L^{-1} KOH	
Content (%)	0.68	0.70	4.90	4.86	4.28	4.64	24.73	24.57
Average (%)	0.69		4.88		4.46		24.65	

Total Se determination by HG-AFS

To determine the total Se concentration extracted with different solvents, a 0.5 mL of the 30 mL supernatants was digested with HNO_3: $HClO_4$(4:1,v/v). All samples were analyzed for total Se by HG-AFS. A detailed description of the procedures used for the determination of total selenium is given in reference[7]. The results of the extraction efficiency were shown in Table 3. Note that the extraction rate was the percentage of Se in the supernatants compared with total Se in 1g yeast.

Table-3: The total amounts of Se extracted and the extraction rates of total Se in different solvents. Two replicates were used.

Solvents	Se concentration in supernatants (μg g^{-1})			Extraction rate of total Se (%)
	Sample #1	Sample #2	Average	Average
70% ethanol	2.48	2.58	2.53	4
Deionized water	9.23	9.11	9.17	15
0.5mol L^{-1} KCl	10.90	10.62	10.76	17
0.1mol L^{-1} KOH	19.98	19.39	19.69	32

Se species determination by HPLC-UV-GC-AFS

The enzymatic hydrolysis method is the best approach to extract different Se compounds, giving a total recovery rate of 90%[8]. A 5 mL of a 30 mM L^{-1} Tris-HCl buffer solution (pH 7.5) was added to 1 mL yeast alkali supernatant. Then the samples were processed in the ultrasound bath for 30 min. 20 mg protease K was added into the samples with incubation at 50 ^{0}C for 18 h. Once again 20 mg protease K was added to hydrolyze completely for 6 h. And finally 40 mg of protease XIV were added and the mixtures were kept at 37 ^{0}C for another 18 h. During the two-enzyme digestions, the sample slurries were constantly and gently homogenized, using a rotary shaker set at 250 rpm. The final hydrolysed samples were centrifuged at 10000 rpm for 30 min. The resulted supernatants were filtered through 0.22 μm filters. An appropriate dilution of these samples was performed prior to their analysis for Se speciation.

To obtain information about the Se species presenting in extracted supernatants, speciation analysis using HPLC-UV-GC-AFS was performed[9]. Each sample was injected into the column, with a 40 m mol L^{-1} phosphate buffer (pH=6.0， flow rate 1mL min^{-1}) as the mobile phase, then the eluent from the column was mixed with 10% HCl (flow rate 3mL min^{-1}) and subsequently passed through the UV unit. 1.2% $NaBH_4$ in 0.35mol L^{-1} NaOH (flow rate 3mL min^{-1}) was added after the UV unit. Argon as a carrier gas (flow rate 300mL min^{-1}) was used to transfer H_2Se from the gas liquid separator into the AFS detector. HPLC-UV-GC-AFS gave detection limits of 27μg kg^{-1} sample for Se^{4+} and 105 μg kg^{-1} sample for SeMet[10]. The calibration curves were established for five independent injections of five different concentrations (20, 40, 60, 80, 100 μ g kg^{-1}). Retention time and regression coefficient (R^2) obtained for each selenium species are summarized in Table 4.

Table-4: Retention time and regression coefficient obtained for each selenium species.

Compound	Retention time (min)	Correlation coefficient (R^2)
$SeCys_2$	2.8±0.1	0.9972
SeMeCys	3.3±0.2	0.9981
Se^{4+}	3.5	0.9996
SeMet	4.4	0.9951

RESULTS AND DISCUSSION

Dependence of protein content and total Se extracted on types of solvents

The KOH solvent seemed to work most efficient for extracting proteins. When the KOH concentration was 0.1mol L^{-1}, the total Se extraction efficiency was 32% which was the highest among all four solvents used in this study. Note that the alcohol soluble was only 4%, while deionizer water and the salt solution gave around 15% and 17% of total Se respectively, of which the difference was not significant. The results were consistent with that from McSheehy S. et al. 2002[11], where water has been used for extracting high and low-molecular-weight species but the extraction efficiency was reportedly low, about 10–15% of the total Se in the yeast.

Based on the results of detecting the protein concentration using the Bradford method, the protein extraction rate with the 0.1 mol L^{-1} KOH solvent accounted for 24.65% of the total proteins. And the results showed that the extracted proteins were mainly alkali soluble. The alcohol soluble gave the extraction efficiency of 0.69% which was almost zero. This indicates that proteins in yeast cells are hardly alcohol

soluble, perhaps due to their hydrophobic nature and the disulphide bonding between protein molecules. It is believed that the high alkaline concentration helps to break down the hydrogen bonds, thus to dissociate the hydrogen atoms from carbolic and sulphate groups. The increased surface charges of protein molecules will then lead to an enhanced solubility in water. Thus, it is obviously seen that alkali solution is the best extraction solvents.

In order to investigate the dependence of the selenium-containing proteins extraction efficiency on the KOH concentration，five different KOH concentrations were used：0.05, 0.1, 0.15, 0.2, 1.0 mol L^{-1}. Table 4 summarizes the data with two replicated sets of samples.

The extraction efficiency of the total Se was shown to increase with the KOH concentration. At relatively low alkali concentrations (<0.2 mol L^{-1} KOH), the extraction rate increased almost linearly with the increase of alkali concentration. With a KOH concentration of 0.2mol L^{-1}, the rate was as high as 60%. However, any further increase of alkali concentration appeared to be less impressive. Only a marginal increase to 68% of the extraction rate was observed when the KOH concentration was 1mol L^{-1}.

Direct species determination of supernatants by HPLC-UV-GC-AFS

It can be concluded from the Table 2 that the proteins in yeast are mostly alkali soluble proteins. Combining with the total selenium (Table 3) extracted, it can be seen that the KOH is the most suitable solvent among the four used. Furthermore as shown in Table 3, when the KOH concentration is 0.1mol L^{-1}, the total Se extraction efficiency is 32%. In order to achieve a much higher extraction rate while keeping the selenium-containing proteins active, the alkalinity should be considered.

The supernatants were filtered through 0.22 μm size filters，and used for Se speciation analysis by HPLC-UV-GC-AFS. The chromatograms of speciation distribution were obtained. The content of Se containing amino acid were caculated by intergrating the peak areas. The speciation distributions of the supernatants are shown in Table 6. Note in Table 6, the organic Se is the sum of $Secys_2$, SeMeCys and SeMet while the total Se has been determined previously as shown in Table 5.

Table-5: Extraction rate with different KOH concentrations.

KOH concentration (mol L^{-1})	Se concentration in supernatants (μg g^{-1})			Extracted rate of total Se (%)
	Sample #1	Sample #2	Average	Average
0.05	16.73	17.65	17.19	28
0.1	19.99	19.39	19.69	32
0.15	23.31	22.50	22.91	38
0.2	38.08	33.72	35.90	60
1.0	43	41.52	42.26	68

From Table 6, we could see that the percentages of organic Se in supernatants with different alkalinity were all quite low, ranging only about 2-11%. It was not clear whether they have already been destroyed in the KOH solutions even with a low alkalinity or they remained in the proteins without being released as the species containing Se amino acid. Thus, we needed to perform enzymatic hydrolysis to have a further study.

Species determination after enzymatic hydrolysis

The species distributions of the supernatants after enzymatic hydrolysis are shown in Table 7.

From the speciation distribution of the supernatants after enzymatic hydrolysis (Table 7), the organic Se containing amino acid and proteins decreased dramatically when the KOH concentration was higher than 0.2 mol L^{-1}. Comparing the results shown in Table 6 and 7, there was obviously much more SeMet after hydrolysis. However, with a 1.0 mol L-1 KOH, it seemed that there was almost no SeMet hydrolyzed out. Furthermore, we found that when the alkalinity was more than 0.2mol L^{-1}, the selenoproteins in the yeast were mostly destroyed based on the ratio of organic Se to total Se. From Table 7, we concluded that the optimal alkalinity was around 0.1-0.15 mol L^{-1} and the organic Se after enzymatic hydrolysis in this condition accounted for nearly 70% of the total Se extracted.

Table-6: The speciation of Se in yeast supernatants before enzymatic hydrolysis was determined by HPLC-UV-GC-AFS. Each KOH treatment had two replicates.

KOH (mol L^{-1})	Secys2 (μg kg^{-1})	SeMeCys (μg kg^{-1})	SeMet (μg kg^{-1})	Se4+ (μg kg^{-1})	Organic Se (μg kg^{-1})	Total Se (μg kg^{-1})
0.05	217	387	0	2886	600	16730
	267	400	212	3379	880	17650
0.10	170	893	0	3645	1070	19990
	314	583	243	5610	1140	19390
0.15	190	495	0	5108	680	23310
	0	1594	781	6534	2380	22500
0.2	322	717	532	6490	1570	38080
	284	728	523	6542	1540	33720
1.0	197	523	200	9987	920	43000
	0	785	0	10396	790	41520

Table-7: The speciation of Se in yeast supernatants after enzymatic hydrolysis was determined by HPLC-UV-GC-AFS. Each KOH treatment had two replicates.

KOH (mol L^{-1})	Secys2 (μg kg^{-1})	SeMecys (μg kg^{-1})	SeMet (μg kg^{-1})	Se4+ (μg kg^{-1})	Organic Se (μg kg^{-1})	Total Se (μg kg^{-1})	Organic Se/ Total Se
0.05	327	488	6097	3607	6910	16730	0.41
	386	431	6256	3768	7020	17650	0.40
0.1	218	453	12766	4037	13440	19990	0.67
	293	464	10114	4968	10870	19390	0.56
0.15	293	365	13774	4826	14430	23310	0.62
	0	474	15478	6315	15950	22500	0.71
0.2	0	373	13225	5847	13600	38080	0.36
	0	356	15559	7508	15920	33720	0.47
1.0	0	592	1316	11894	3210	43000	0.07
	0	408	0	14346	410	41520	0.01

Alkalinity and speciation

The percentage of the organic Se containing amino acids determined directly was low，but it increased dramatically after enzymatic hydrolysis except in the case of the KOH concentration at 1mol L^{-1}. Comparing the protein hydrolysates before and after hydrolysis shown in Table 6 and 7, the dramatic increase in percentage of the organic Se was attributed to the presence of SeMet. Thus, it is evident that SeMet is present in proteins. With an increasing alkalinity, the extracted amount of SeMet increased. However, when the KOH concentration was 1.0mol L^{-1}, the extracted amount of SeMet was small and there was little change before and after hydrolysis. This demonstrated that selenoproteins were destroyed and did not combine to form Se amino acid in a high alkalinity.

In addition, the amounts of SeMeCys were shown decreasing after hydrolysis almost in the each experiment except the KOH concentration was 0.05mol L^{-1}. Based on this phenomenon, we believed that SeMeCys in the supernatants were unstable. There were little changes to $SeCys_2$ before and after hydrolysis. The absolute amounts increased a bit after hydrolysis but contributed very little to the total organic Se increase. In a summary, we can conclude that the stability of these three kinds of Se amino acids has the following relationship: SeMet> $SeCys_2$> SeMeCys.

In contrast to rice，the achieved protein extraction efficiency here were not very high. Guo et al[12] achieved 55% protein extraction from rice using a 0.1 mol L^{-1} NaOH solution.. Thus, we suspected that our low extraction efficiency was attributed to the ultrasound technique used. The yeast cell walls composed of polysaccharides were hardly alkali-soluble. It is believed that in order to study the optimal extraction

method one would need to find a better way to rupture cell walls furthermore.

Among the samples analyzed especially between the two replicated sets, there were some derivations in the results. This may be attributed to the fact that yeast is a product of biological engineering. It is very likely to have a certain amount of variation with respect to its Se content and speciation. Furthermore, it is still technically challenging to have a full identification of all the Se species that are contained in Se yeast[13]. Its metabolism is much more complex than plants.

CONCLUSIONS

This work demonstrated that alkali solvents could be used effectively to extract the selenium-containing proteins, and after the extraction process, the main amino acid was SeMet which were combined in the proteins. The speciation studies revealed that when the selenium-containing proteins exposured in the high alkalinity, it would be oxidized easily. In such a case with the KOH concentration of more than 0.2 mol L^{-1}, the amino acids and proteins would decrease dramatically. In order to extract selenium-containg proteins effectively, the alkalinity of solvents should be kept moderate to ensure the protein integrity. The optimal alkalinity was shown to be in a range of 0.1-0.15 mol L^{-1}. In this condition the resulted organic Se after enzymatic hydrolysis accounted for nearly 70% of the total proteins.

Due to the limitation of the technique used in fragmenting yeast, it is believed that the extraction efficiency still has not been achieved at a desired level. With this study, It was confirmed preliminarily the above-stated optimal alkalinity range. The optimal extraction process will require further studies in the future, such as, on how to deal with the cell walls.

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REFERENCES

1. J.W. Finley, J. Agric. Food Chem. **46**, 3702. (1998).
2. P. Moreno, M.A. Quijano, Analytica Chimica Acta. **524**, 315(2004)
3. M.P. Rayman. British Journal of Nutrition. **92**, 557(2004).
4. L.q. Shen, x.y. Wang, Food Chemistry, **107**, 929(2008)
5. T. Zhang, Inner Mongolia University of science and technology Master Dissertation, (2008)
6. N.J. Kruger, The protein protocols handbook, **Part 1**, 15(2002)
7. Y.h. Liu , w.b. Yang, Environmental Science Survey, **28**, 80(2009)
8. D. Mazej, I. Falnoga, Talanta, **68**, 558(2006)
9. P. Cuderman, L. Ozbolt. Food Chemistry, **123**, 941(2010)
10. S. McSheehy, F. Pannier, J. Szpunar, M.Potin-Gautier, Analyst, **127**, 223(2002).
11. R. Guo, S. Pang , K. Wang , Shiping Kexue, **26**, 173(2005).
12. H. Chassaigne, , CC. Cherry, Journal of chromatography, **976**, 409(2002)
13. C. Casiot, J. Szpunar, Journal of Analytical Atomic Spectrometry, **14**, 645(1999)

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