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A Study on the Extraction and Purification of Glutathione from Yeast

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During the course of studies on the production of glutathione from yeast, process development and optimization was carried out. The optimal pH for the extraction of glutathione was found to be 2.5 to 4.0 and the maximum yield for glutathione was obtained when the extraction temperature was 25 to 45°C. The cuprous salt of glutathione was recovered maximally at the range of 2 to 4g of cuprous oxide per 10 Kg of compressed yeast.

Further purification was needed for the removal of impurities from glutathione. Cation exchange resin, anion exchange resin and Sephadex G-25 were employed for this purpose.

13 to 15 g of glutathione was obtained from 10 Kg of compressed yeast and the purity was above 99.3%.

Since Hopkins develped the method for the preparatione of glutathione (GSH) from yeast, several workers have published modification of the original method especially for the extraction and purification of glutathione from yeast.

Among them, Pirie developed a very much improved method with a new solvent system (ether:ethyl alcohol: water) for the extraction of glutathione. Unlike to this, the extract obtained on boiling yeast in water which is common in Japanese patent, was found to give an unsatisfactory cuprous salt when cuprous oxide was added directly to the acidified filtrate.

In this report, the process of extraction and purification for glutathione was studied and checked for the adoptability for industrial scale.

For the extraction of glutathione Pirie method was adopted and modified.

In the previous works,[1-4] the separation of yeast and cuprous salt from extraction solvent was carried out by filtration. To adopt this process to industrial scale is not advantageous, because of long time treatment.

To choose centrifuge for this process saved much time and was concluded as advanta-

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geous process for industrial scale.

Cuprous oxide was used in previous works[1-4] for the precipitation of glutathione from extraction solvent. Cuprous oxide was added for several times and when about half the cuprous oxide (total 1 g for 1 Kg of compressed yeast) was added, the salt should be allowed to settle and the mother liquor poured off for further addition of cuprous oxide. But this method is very tedious and is not adoptable for industrial scale. It was observed that the amount of cuprous oxide added to the extraction solvent which contains solubilized glutathione may be critically calculated from experimental data and added to the extraction solvent in a time. But the extra addition of cuprous oxide caused the resolution of some of the cuprous glutathione compound already formed.

The previous methods[1-4] were not satisfied fully for the purification of glutathione, because the purity of the product was not consistant and the impurity contaminated in cuprous salt was not discarded satisfactory level.

For the production of glutathione in industrial scale, it is advantageous to add the process of ion exchange and molecular filtration.

Beside of these, several processes were developed and optimized.

EXPERIMENTAL

Apparatus——Liquid chromatography was carried out with LKB glass chromatography columns and LBK Varioperpex pump(model 2120). Fractionation was tried with fractional collector(MRK type 4-87TV). Centrifuger employed was Tominaka model CF380F. IR & NMR spectroscopy was carried out with Perkin Elmer IR Spectrometer (model 467) and Varian NMR spectrometer (model HA-100), respectively.

Materials——Cuprous oxide, sulfuric acid, sodium acetate, sodium chloride, ethanol and ether were used in reagent grade. Compressed yeast was purchased from Cheil Universal Co. LTD.

Organism——The organism employed throughout this investigation was *Saccharomyces cerevisiae* purchased as compressed yeast.

Extraction Solvent——The extraction solvent was composed with ethanol; 1L, ether; 0.8L and H_2O; 2.5L. pH was adjusted from 3.0 to 4.0 with H_2SO_4.

Extraction of Glutathione from Yeast——10Kg of compressed yeast was suspended into the above extraction solvent and blended thoroughly until almost all of the clid disintegrated into emulsion type. Then fix the temp. to 25~35°C for 30~60 minutes. After heat treatment, yeast cell was quickly cooled and separated from extraction solvent with centrifuger (Tominaka model CF 380F) for 30 min. at 8,000 rpm. Yeast was suspended again into 7L of 1% of sulfuric acid and centrifuged at the same condition. The supernatant was colleted.

Reaction with Cu_2O——The supernatant collected was heated to 20~50°C. Then cuprous oxide was added to this solution and reacted for 20~60 minutes. The precipi-

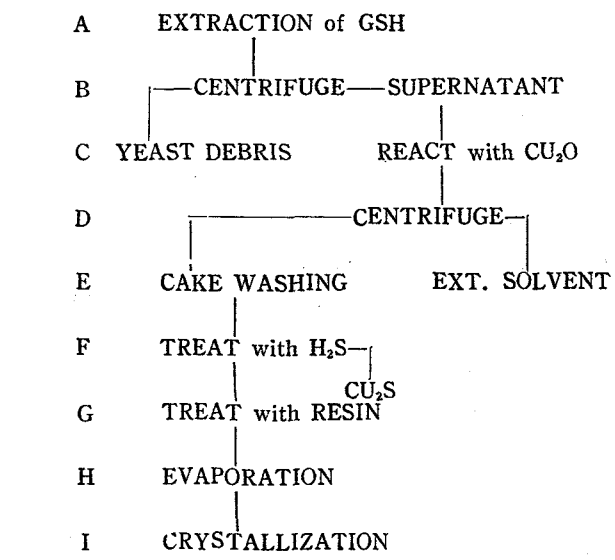
tate was centrifuged and washed until the precipitate was free from sulfates. Then the precipitate was suspended in water. Hydrogen sulfide was added by bubbling the gas through the liquid containing the copper glutathione compound. The copper was precipitated as the insoluble copper sulfide, the glutathione remaining dissolved in the filtrate. The precipitate of copper sulfide was filtered off and the filtrate was evaporated under vacuum.

Purification of Glutathione from Column Separation——After the filtrate was evaporated, further purification was carried out by treatment with cation exchange resin, anion exchange resin and Sephadex, sequentially with 0.1 M acetate buffer (pH 3.0). Fractionation was tried with fractional collector (MRK type 4~87TV). Scheme 1 shows the total process.

Assay for Glutathione——Glutathione was assayed with 0.1 N I_2 solution after the process F in Schemel.

Identificiaton of Glutathione——Crystallized glutathione was sampled for IR and NMR. IR chart was obtained from Perkin Elmer IR spectrometer (model No. 467) in KBr disc. NMR spectrum was obtained in D_2O at 100 MHz from Varian NMR spectrometer (model HA-100).

Scheme 1. Extraction & purification process for glutathione (GSH)



RESULTS AND DISCUSSION

Effect of pH on GSH Extractability——The extractability of glutathione at different pH values was determined. The pH range covered was from 1.5 to 11.5. The extraction was carried out with the extraction solvent containing modified Pirie solvent

As shown in Fig. 1, maximum extractability was obtained within the pH range of 3.0 to 4.0 The extraction was severely diminished under pH 2.0

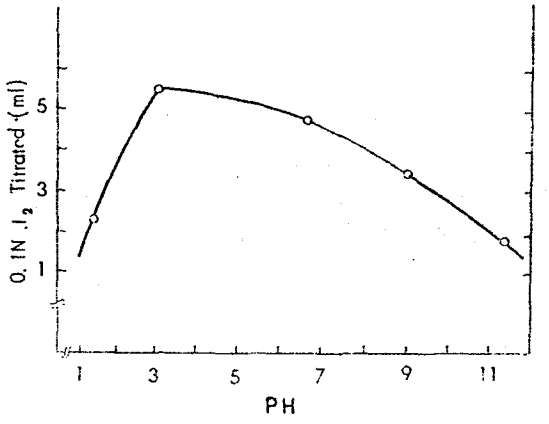


Figure 1—Effect of pH on GSH extractability
Ext. Solvent; Pirie Reagent
Ext. Time; 30~60min
Ext. Temp.; 20~40°C
pH: Adjust with H_2SO_4 and NaOH
Assay for GSH;Titrate with 0.1N I_2 after the process

Effect of Extraction Temerature on the Extractability of Gluthione and Viscosity——The extraction was carried out with the extraction solvent containing modified Pirie reagent. Viscosity was also measured at same time.

Effect of pH & Temperature on the Activity of Glutathione after Storage for 16 Hrs——Glutathione activity was studied after storage for 16 hrs at two different temperature and different pH values. The pH range covered was from 2.0 to 10.0. The result is shown in Table I. The activity of glutathione was lowered exponentially with the increase of pH. The effect of temperature on the activity of glutathione at each pH values is also showning Table I. The effect of temperature on the a activity of glutathione at 0~5°C was much less than that at 20~25°C and the subtraction of the two activity was gradually increased with the elevation of pH.

Table I—Effect of pH and Temperature on GSH Activity After Incubation for 16 hrs

Activity* Loss(%) / Temp.	pH				
	2.3	4.2	6.0	8.4	10.2
0~5°C	2.5	5.0	10.8	12.4	61.0
20~25°C	13.0	18.5	39.4	79.5	—

* Assay for GSH: Titrate with 0.1N I_2.

Effect of the Amount of Cuprous Oxide on Gutathione Yield——Glutathione was separated from extraction solvent with cuprous oxide at different amount of cuprous oxide. Reaction was carried out at 20~50°C for 30~60 minutes. As shown in Fig. 2, the optimal amount of cuprous oxide for the reaction was 2.5 to 4.0g for 10Kg of compressed yeast. Below the optimal amount of cuprous oxide, inhibitory effect of a certain component in the extract was observed, whereas above the optimal amount of cuprous oxide also shows negative effect on salt formation of copper with glutathione.

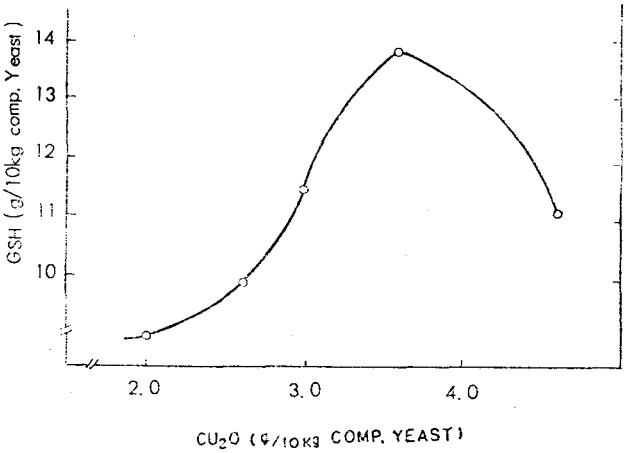


Figure 2—Effect of the amount of Cu_2O on GSH yield
Rexn Temp.; 20 50°C
Rexn Time; 30 60 min
Assay for GSH; Titrated with 0.1N I_2 after process F.

As shown in Table Ⅱ, maximum extractability of gluathione from yeast was obtained within the temperature range of 25-35°C. When the temperature was under 25°C, the extractability was abruptly downed, whereas above 35°C measurable glutathione was diminished, probably because of the destruction of glutathione by heat treatment. Over a mide range of temperature, glutathione was extracted in a full scale of extractable glutathione. In view of viscosity, the temperature range 5 of 25 to 30°C shows the lowest viscosity and probably this temperature range must be adopted as extraction temperature for industrial scale, because as shown in Table Ⅱ, above 39°C, the viscosity increases gradually and high viscosity is not good industrial process such as filtration and centrifugation.

Separation of Glutathione on Cation Exchange Resin(R-COO Form)——Glutathione sample was loaded on cation exchange resin after treatment with cuprous oxide and evaporation. The flow rate was 3.5ml per centimeter square per minute. The inner diameter was 9 milimeter and the bed height was 30 centimeter. Sample loaded was corresponding to 0.5g of pure glutathione. Concentration gradient was made with

Table Ⅱ—Effect of Extraction Temperature on the Extractability of GSH and Viscosity

Ext. Temp.	20	25	30	35	40
GSH*	11.2	13.9	14.0	14.1	9.3
Viscosity	29	20	21	35	44

Assay for GSH ; Titrate with 0.1N I_2 after the process F.
Viscosity ; Measured with viscotester (Model VTOI: Rion Co. Ltd.) at 20C
* Gram of GSH per Kg of comp. yeast

sodium chloride. The elution buffer was fractionated into 5ml and glutathione fraction was collected. The elution profile is shown in Fig. 3. Several impurity peaks were observed during elution to the extent of 300ml. Thus, glutathione collected was loaded to anion exchange resin.

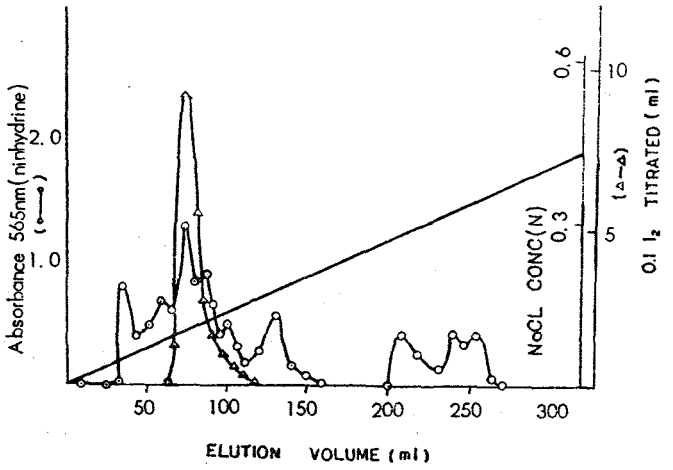


Figure 3—Separtion of glutathione on cat. ex.
Resin (R-COO^- form), 9mm×30cm.
Flow rate; 3.5ml/Cm^2/*min.*
Elution buffer; 0.1M acetate buffer (pH 3.0)

Elution of Glutathione from Column of Anion Exchange Resin (R−$N^+(CH_3)_2$ Form)——After loading of glutathione to anion exchange resin, the column was washed and fractionated with acetated buffer (pH 3.0). All of the condition was the same as that of cation exchang resin. Concentration gradient was also made with sodium chloride. Elution profile for anion exchange resin is shownn in Fig. 4. It was observed that almost all of the impurities were removed, but further purification must be needed for pure glutathione. Thus, the glutathione fraction was collected and evaporated.

Purification of Glutathione on Sephadex——Sephadex G-25 was adopted for the molecular separation of glutathione and the impurities. Inner diameter of the column was one centimeter and the bed height was 60 centimeter. Flow rate was 4.1ml per

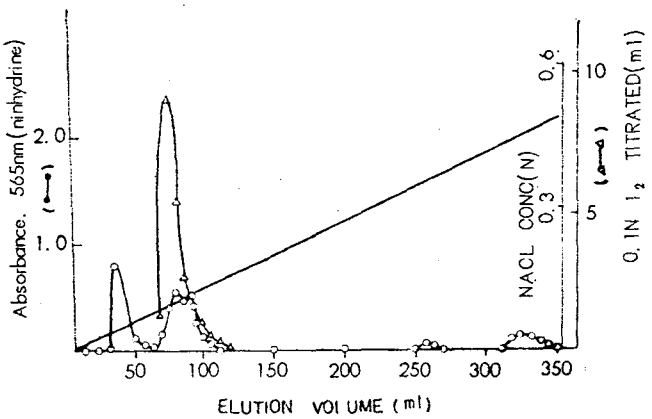


Figure 4 - Elution profilre from column of anion ex. resin ($R\text{-}N^{+}(CH_3)_2$
9mm×30cm
Flow rate; 3.5ml/cm²/min.
Elution buffer; 0.1M acetate buffer (3.0)

centimeter square per minute. Elution buffer was 0.1M acetate buffer at pH 3.0. Elution profile is shown in Fig. 5. As shown in Fig. 5, negligibles were observed after this process. After the glutathione fraction was collected, the solution was concentrated until glutathione was saturated.

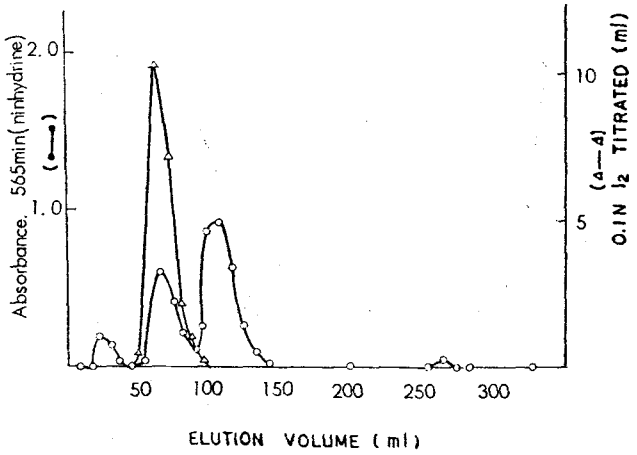


Figure 5 - Purifcation of glutathione on Sephadex G-25 1cm×60mm
Flow rate; 4. 1ml/cm²/min
Elution buffer; 0.1M acetate buffer (pH 3.0)

Crystallization of Glutathione——The saturated soluton was taken up in 50% ethanol and cooled to 0—5°C. The glutathione was crystallized out after several days and removed by filtration. 13 to 15g of glutathione was obtained from 10kg of compressed yeast and the purity was above 99.3%.

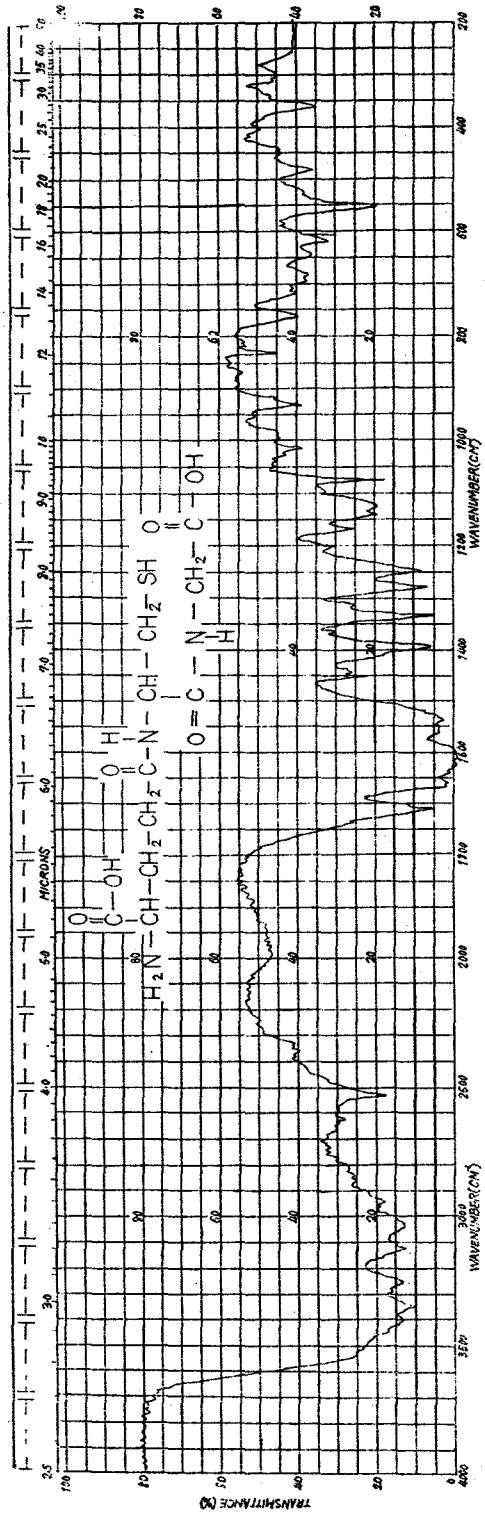


Figure 6 - IR spectrum for glutathione

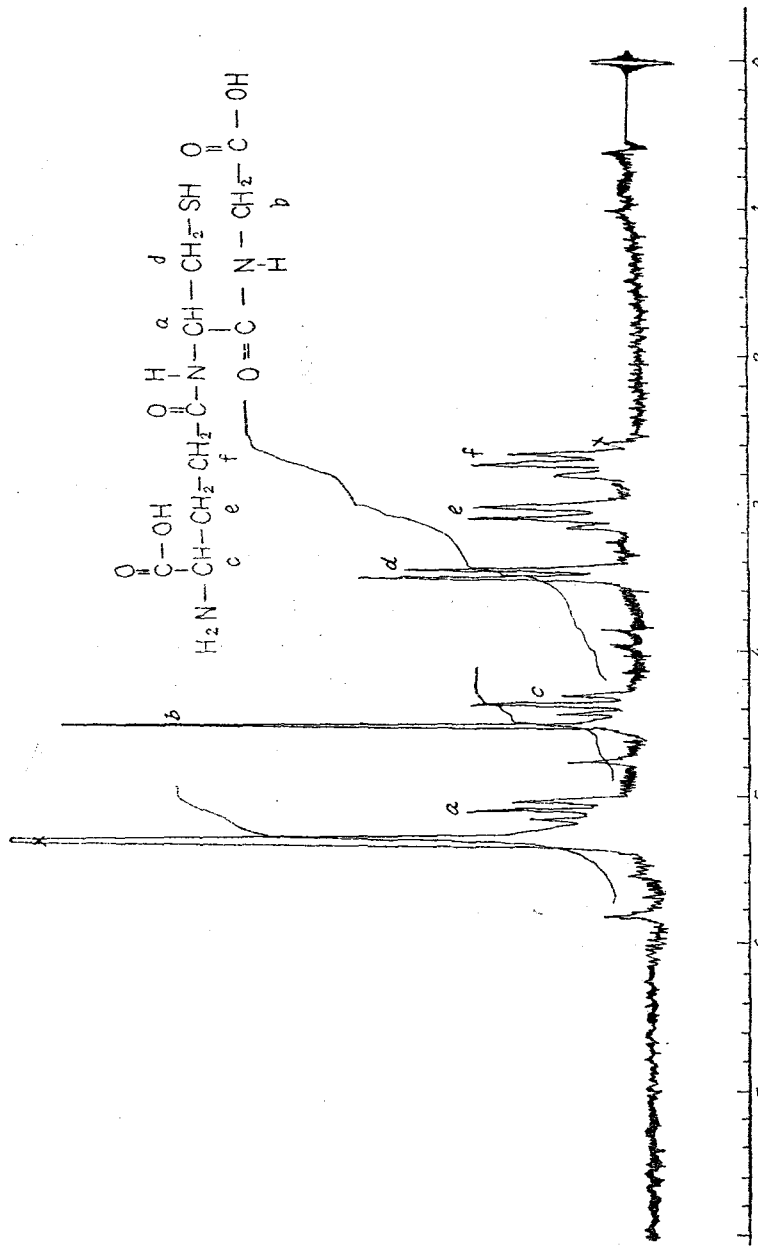


Figure 7 - NMR spectrum for glutathione in D_2O at 100 MHz

IR Spectrum for Glutathione——IR spectrum was obtained after crystallization. The result is shown in Fig. 6. Fig. 6 shows that, stretching vibration of imide in peptide bond at 3340 and 3260cm^{-1}, sulfidryl group at 2,530cm^{-1}, carboxy group at 1710, 1655, 1395, 1280 and 1250 cm^{-1}, and bonding vibration of -CH_2- at 1450 and 1330 cm^{-1}, carboxy group at 930cm^{-1} and imide in peptide bond at 1530 cm^{-1}, revealed clear abssorption at respective wave number. This peak may be used for identification of glutathione.

NMR Spectrum for Glutathione——NMR spectrum was obtained with crystallized sample. Signals corresponding to each group are shown in Fig. 7. From Fig. 7, it was observed that sulfydril carboxy and imide group showed no clear peak in the given range.

CONCLUSIONS

The extraction and purification of glutathione from yeast was studied. The optimal pH for the extraction of glutathione was found to be 2.5 to 4.0 and the maximal yield for glutathione was obtained when the extraction temperature was 25 to 45°C. The cuprous salt of glutathione was recovered maximally at the range of 2 to 4g of cuprous oxide per 10kg of compressed yeast. Further purification was carried out with cation exchange resin of R-COO^- form, anion exchange resin, of R-N^+ $(CH_3)_2$ form and Sephadex G-25. 13 to 15g of glutathione was obtained from 10 Kg of compressed yeast and the purity was above 99.3%.

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