

Research Report

Identification and Purification of <Geranti Bio-Ge Yeast>

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Retranslated into English: Aug., 2004

The National Instrumentation Center
for Environmental Management,
College of Agriculture & Life Sciences,
Seoul National University, Korea

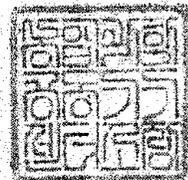
Identification and Purification of <Geranti Bio-Ge Yeast>

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Researcher : Head researcher, Prof. Eun Woo, Park



Introduction

<Geranti Bio-Ge Yeast> is a newly invented product, which contains organic germanium by biosynthesis of yeast and germanium.

Such type of organic Bio-Germanium Yeast is a substance which is made by interacting of biomolecules like proteins, nucleic acid or organic acid and inorganic form of germanium during the biosynthesis technology.

Since anti-tumor activity of germanium (Ishida et al, 1979), anti-mutation activity (Li et. al., 1982, Kada et. al, 1984) etc., researchers were very interested in biosynthesis of microorganism and inorganic germanium in order to make use as a health substance.

Klapcincka et. al. reported in 1986 that Ge in *Ps. putida* cell is mostly accumulated in soluble fraction, and is bound with nucleic acid and proteins by their electron microscope study. And Wei et. al. reported that 'yeast containing organic germanium' can be produced by biosynthesis technology (1992).

Lynn M. Ramsay & Geoffrey M. Gadd reported that 'Mutants of *Sacchromyses cervisae* defective in vacuolar function a role for the vacuole in toxic metal ion detoxification' (Microbiology Letters, 1997), ie: yeast transforms inorganic germanium into organic bio-germanium by binding with Ge, and detoxifies toxicity of inorganic germanium by its metabolism.

The purpose of this study are to confirm that 1) <Geranti Bio-Ge yeast> has been transformed into organic form during biosynthesis process in fermentation, and 2) its purification and identification.

1. Materials and Methods

1) Material

'Geranti Bio-Ge yeast' (3,275 ppm grade) which has been made by Geranti Pharm Ltd.

2) Extraction

3g of 'Gerant Bio-Ge yeast', freeze-dried by nitrogen, was comminuted by bowlmill. The powder of 'Gerant Bio-Ge yeast' was been in suspension by 4-5 ml of 50 mM Tris-HCl (pH 7.5) buffer, and then the mixture was centrifuged for 20 min. at 12,000 rpm. Taking only this soluble supernatant and then put to filtration by 0.45 um filter to eliminate impurities. By thus, the sample was prepared for the study of 'purification and identification' of 'Geranti Bio-Ge yeast'. This filtrated and purified supernatant is called 'Geranti Bio-Ge yeast Extract' throughout this report.

3) Determination of Germanium

ICP analyzer (SHIMADZU ICPS-1000) in NICEM was used for this study. The condition of analysis was 209,49 nm, R.F. Power 1.2kw, and torch height 15mm. Aldrich Ge standard solutions was used.

4) SDS- PAGE Analysis

The purity and molecular weight of Ge-binding protein were determined by Coomassie blue SDS-PAGE. The gel was prepared by mixing with 7.5ml of 30% acrylamide, 3.5ml of Tris-HCl pH 8.8 and 3.75ml of H₂O, and eliminating bubbles under vacuum condition. And then, put 0.05 ml of ammonium sulphate and 0.01 ml of TEMED to gel plate and solidificated. After loading this gel, let it run about 1 hour at 300v 15mA. Analyzed the band after decoloration.

5) Ion Exchange Chromatography

Liquid Perfusion Chromatography (Bio-Rad System), owned by The National Instrumentation Center for Environmental management, College of Agriculture & Life Sciences, Seoul National University, was used for this study. Ion Exchange Chromatography was carried out on Poros column HQ-20 and Poros HS-20, and buffer A is 50 mM Tris-HCl (pH 7.5), elution buffer B is 50 mM Tris-HCl (pH 7.5) and 1 M NaCl were equilibrated at a flow rate of 4 ml/min. Washed about 5 min. by buffer A, and then washed 5 min. by buffer B, and then 5min.

with buffer A. The protein come out of column, each fraction 4 ml, was monitored by UV-absorption at 280 nm.

6) Gel Filtration

Gel Filtration was carried out on SHODEX Protein column KW-803 (8mm × 300mm, exclusion limit 1.5×10^5 equilibrated with 50 mM Tris-HCl (pH 7.5) buffer and eluted at a flow rate of 0.5 ml/min. Washed column for 15-20 min. After injected 'Bio-Ge Yeast Extract' to the column for 40 min. And then, took the proteins (1 ml per each fraction) come out of the column and were monitored by UV-absorption at 280 nm.

7) Prep. Cell purification

The purification of Ge-binding protein were carried out by prep cell unit (Bio-Rad co.) on a gel prepared with 5% acrylamide and run for 3 hours at 40mA with Tris-glycine buffer (pH 8.3). Electrophoresis buffer was Tris-glycine buffer(pH 8.3). For fraction of protein, sample was eluted at 1 ml per fraction.

8) N-terminal sequencing

N-terminal sequencing was analyzed according to the Edman procedure by 491 Protein sequencer (Applied Biosystems) owned NICEM. Prior to sequencing, Ge-binding protein obtained by

electrophoresis was transferred to PVDF membrane at 20V for 90 min. The protein transferred PVDF membrane was dyed into Coomassie brilliant blue. polyacryamide gel were electroblotted onto a PVDF membrane (sigma, USA), followed by staining with Coomassie brilliant blue.

2. Results

The isolation and purification of 'Geranti Bio-Ge yeast' has been done as per Scheme 1. The result from each step is as followings:

1) Ion Exchange Chromatography

Ion exchange chromatography was conducted to eliminate proteins that were not bound with germanium (Fig. 1 & 2). 'Geranti Bio-Ge Extract' were loaded in anion and cation columns. The fraction which was estimated as Ge-binding protein was not bound in anion and cation column.

First, cation exchange chromatography was done, and then, collect the eluted fractions from cation exchange chromatography, and then, do anion chromatography again to collect eluted fraction (#1-3 in Fig. 2).

When 'Geranti Bio-Ge Extract' conducted in anion and cation chromatography, there were some proteins bound with anion and cation, but Ge was not detected in the fraction that was bound in column (#9-11 in Fig. 1, 2.). This result shows that 'Ge-binding protein' is not bound in ion exchange column according to its characteristic.

2) Identification of organic germanium

The result of gel filtration of 'Geranti Bio-Ge yeast Exact', both protein and germanium were detected in the fraction No. 12, 13. So we can assure the presence of Ge-binding protein in the fraction No. 12, 13.

When analyzed the sample of fraction No. 12, 13 concentrated and dried by SDS-PAGE, several protein bands were shown (data no shown). Therefore, the result of Fig. 3 suggest two interpretations as followings:

- 1) There are(is) Ge-binding protein(s) in these fractions.
- 2) Under the experimental conditions, ionized germanium peak which does not bind to a protein(s) happen to be coincident with these fractions (No. 12, 13).

In order to investigate above two cases, the following experiments were carried out.

Experiment-A:

Gel filtration of the extract of Yeast that was cultured without germanium source, i.e. inorganic germanium(GeO_2), and compared this with Fig. 3. If Ge-binding protein(s) be present, protein peaks will be conform to Fig. 3 but there will be no peak for germanium.

Experiment- B:

Gel filtrated the mixture of <Geranti Bio-Ge yeast Extract> that was cultured with inorganic germanium(GeO_2) and inorganic germanium. If there is organic germanium in <Geranti Bio-Ge yeast Extract>, we can find the peak of Ge-binding protein as well as GeO_2 .

Experiment- C:

Gel filtrated only the inorganic germanium and checked fraction of it under the same conditions, and then compared this with Experiment-B. We can confirm that there is (are) Ge-binding protein by this result.

Fig. 4 shows the result of gel filtration of yeast extract cultured without GeO_2 . The elution profile of protein detected by UV-

absorption at 280nm is in accordance with Fig. 3, but there was no germanium peak in fraction 12 & 13 by ICP.

And the result of gel filtration of <Geranti bio-Ge yeast Extract> and GeO₂ mixed, GeO₂ is in fraction No. 20-30 and Ge-binding protein is in the fraction No. 12-13. Therefore, we could confirm that <Geranti bio-Ge yeast> is bound with protein and germanium(Fig. 5). Inorganic germanium (GeO₂) with dissociated form is in fraction No. 20-30 indifference with existence of protein.

We could also confirm that Ge in fraction No. 12-13 is that of Ge-binding protein by these research, so we have done prep cell purification to know the Ge-binding protein(s).

3) Isolation of Ge-binding protein

In order to do isolation and purification of Ge-binding protein in fraction No. 12-13, Prep cell analysis has been done. As result, Ge-peak was demonstrated in fraction No. 13-15(Fig. 7).

Concentrated and freeze-dried this fraction analyzed by SDS-PAGE. We could find out that this was correctly isolated and purified, and its molecular weight is about 34,000 Da.

SDS-PAGE (Fig. 8). Lane 8 demonstrate putative Ge-binding protein.

4) N-terminal Sequencing of Ge-binding protein(s)

In order to know the kind of protein in Ge-binding protein, SDS-PAGE ran to obtain PVDF membrane. Taking out the membrane from band, and have done N-terminal sequencing. Compared this result with that of already known protein in yeast are as Table 1.

(N-terminal sequences for the 34 KDa protein are in Table 1. We named the putative Ge-binding protein as GY-45)

Table 1. N-terminal sequences for the putative Ge-binding protein (GY-45) and those of sequence-matching protein that was found by BLAST search.

No.	1	2	3	4	5	6	7	8	9	10
GY-45	A	V	S	K	V	Y	A	R	S	V
Enolase	A	V	S	K	V	Y	A	R	S	V

By using BLAST program, searched Ge-binding protein. NCBI data base of *S. cerevisiae* was used. The search resulted in five proteins; The N-terminal sequences of GY-45 were matched with The search resulted in five proteins; EN02_YEAST, EN01_YEAST from Swiss data bank, 171455 from GenPept, NOBY2 and NOBY from PIR.

Among these five proteins, the first three (ENO2_YEAST, EN01_YEAST, GenPept) are known as enzyme Enolase and the other two (NOBY2, NOBY) are phosphopyruvate hydratase.

Especially, the first ten N-terminal sequences of Enolase was perfectly matched with those of GY-45. Enolase is known as an enzyme that transfer 2-phosphoglycerate into phosphoenlpyruvate, and has active site that can bind metal ions like Mg and Ca in amino acid residue.

Therefore, this suggests that Ge can be bound with enolase.

3. Conclusions

According to this study, we could confirm that <Geranti bio-Ge yeast>, invented by Geranti Pharm Ltd.in 1993, is bound with Enolase proteins and Ge by biosynthesis technology.

N-terminal amino acid sequences using a purified Ge-binding protein have a homology with a metal-binding protein Enolase.

4. Further research, suggested

We suggest further studies in molecular biology to find the gene that bind with Ge, and also the mechanism of binding protein with Ge.

References

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Scheme 1. Steps in purification of Ge-binding proteins

Dried Yeast-G(Bio-Ge) extract



Ion Exchange Chromatography



Gel Filtration Chromatography



Prep. Cell Purification



N-terminal sequencing

Fig.1. Elution profile from cation ion exchange chromatography for <Geranti Bio-Ge Yeast Extract>. Fractions with Ge activity were clearly seen in the fraction number 1-3 which was measured by using ICP.

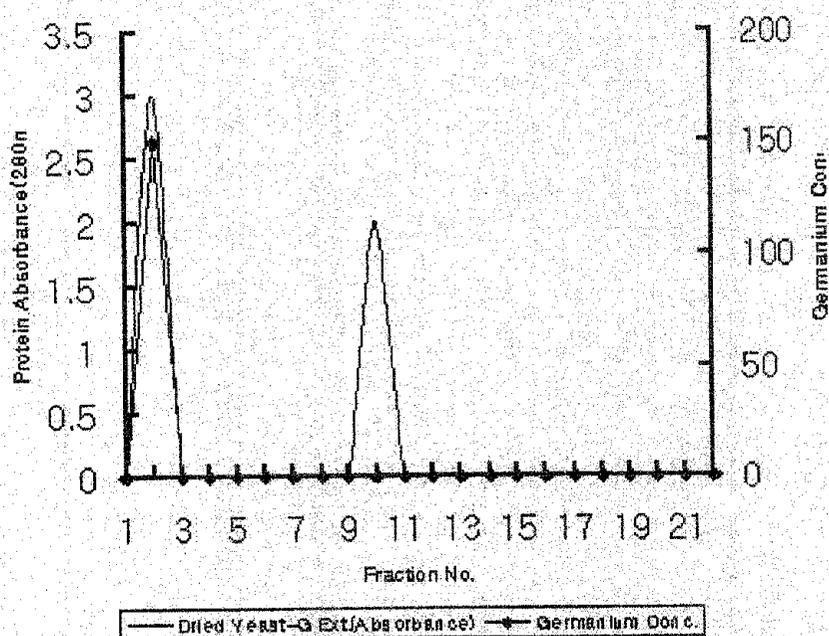


Fig.2. Elution profile from anion ion exchange chromatography for fraction 1-3 in Fig. 1. As shown in Figure 1, fractions with Ge activity (#1-3) were not bound to the column.

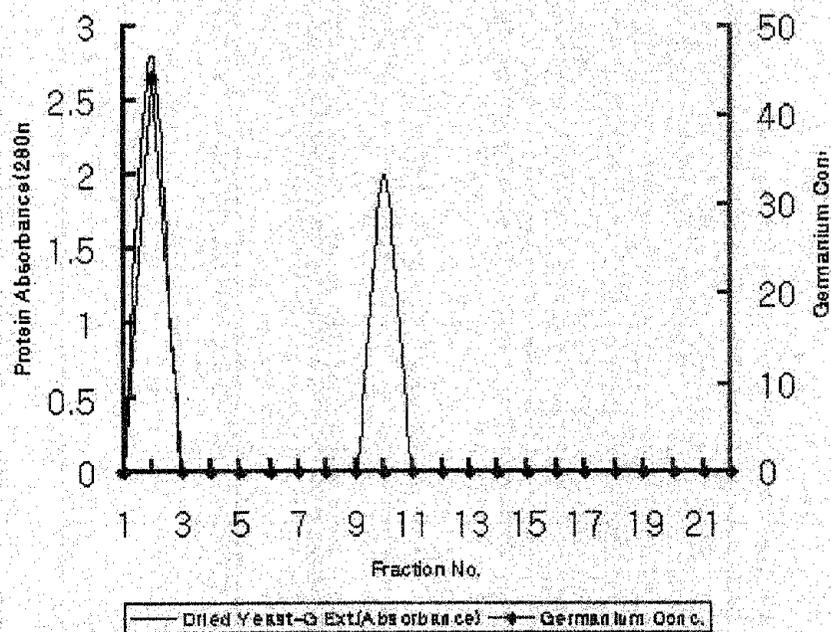


Fig.3. Elution profile of gel filtration (Shodex protein KW-803 column) of <Geranti bio-Ge yeast extract>. Proteins in fractions 12-14 are expected to be Ge-binding proteins as indicated with high concentration of Ge.

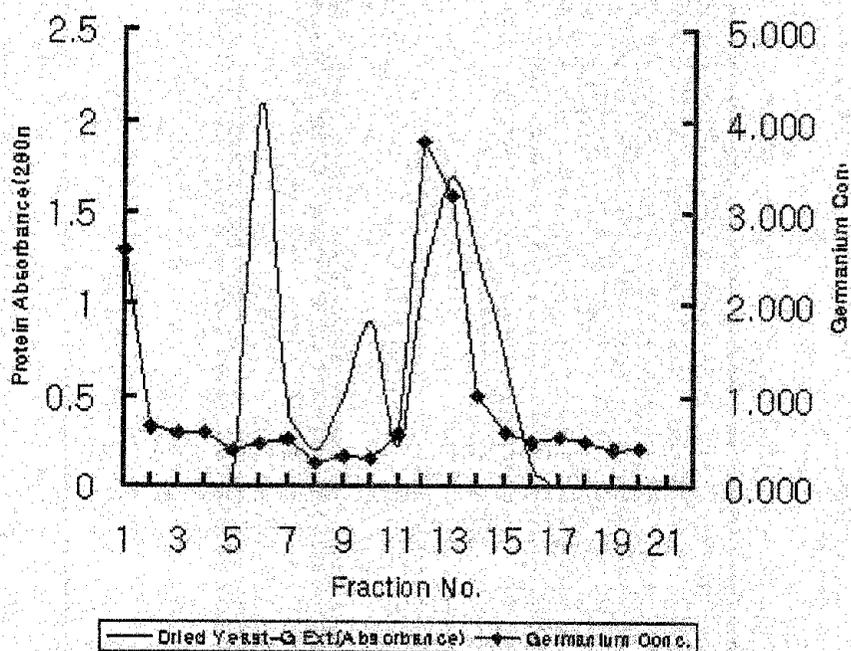


Fig.4. Elution profile of gel filtration (Shodex protein KW-803 column) for yeast extract. Yeast were cultivated in the absence of GeO₂. Unlike Figure 3, there are no trace of Ge for the fractions 12-14.

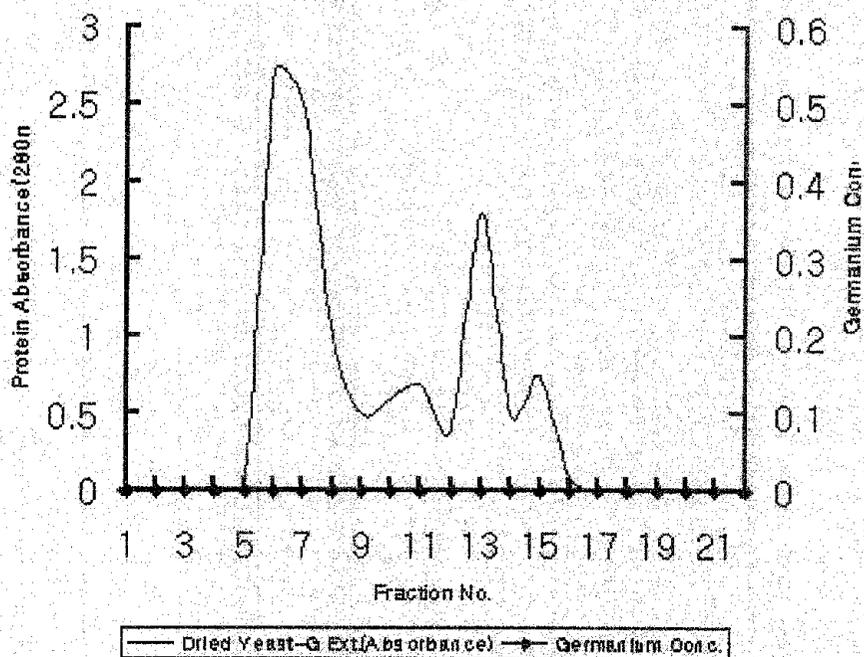


Fig.5. Elution profile of gel filtration (Shodex protein KW-803 column) for the mixture of yeast extract and GeO₂. Yeast extract from ion exchange chromatography were mixed with free GeO₂. Due to difference in Ge relative activity between bound form and free form, it shows very low concentration of Ge in the fractions 12-14 and high concentration of Ge in the fractions 20-24.

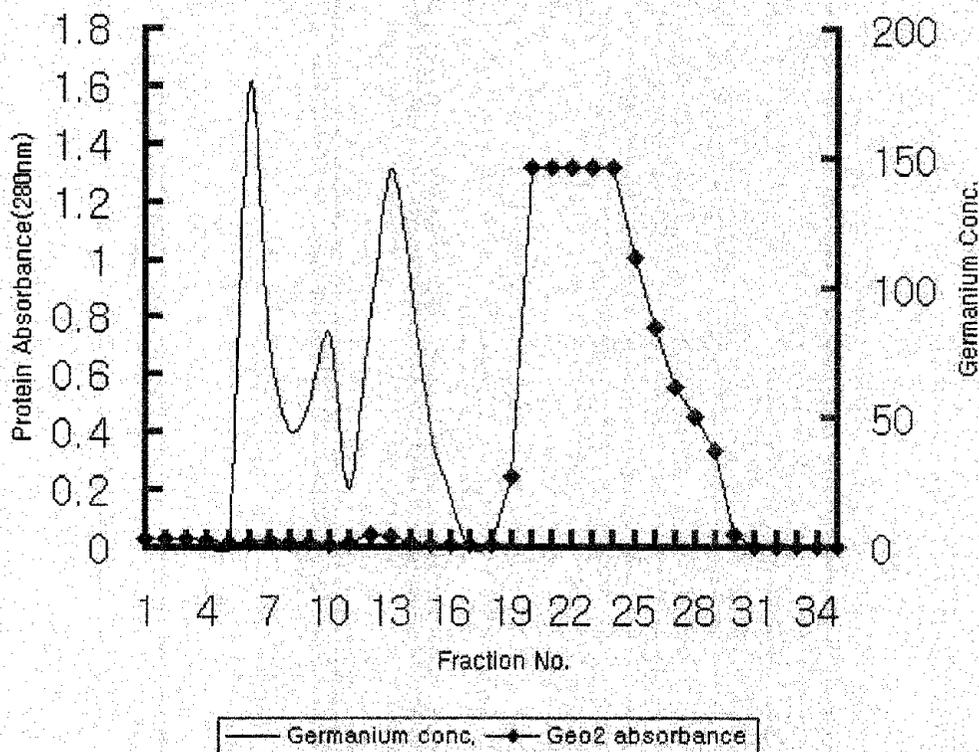


Fig.6. Elution profile of gel filtration (Shodex protein KW-803 column) of inorganic germanium (GeO_2). Ge peaks were found at the fractions 20–24. It suggests that fractions 13–14 in Figures 3 and 5 are that of Ge bound form to the proteins.

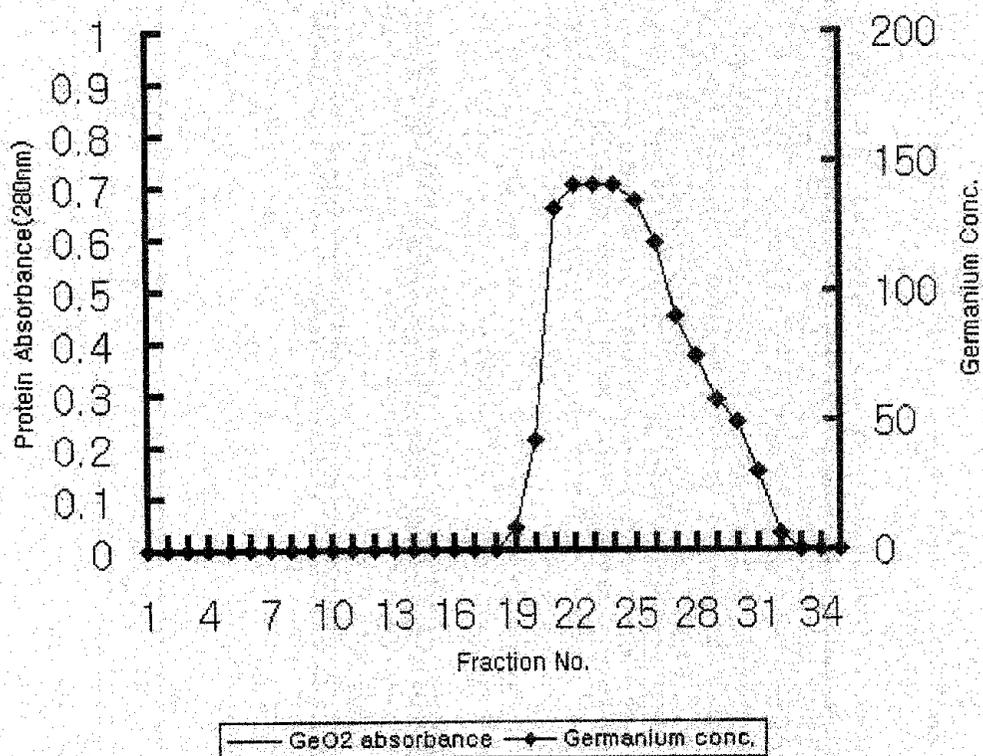


Fig.7. Germanium content of each fraction separated by prep cell. Fraction with high concentration of Ge correspond to MW of 34 KDa.

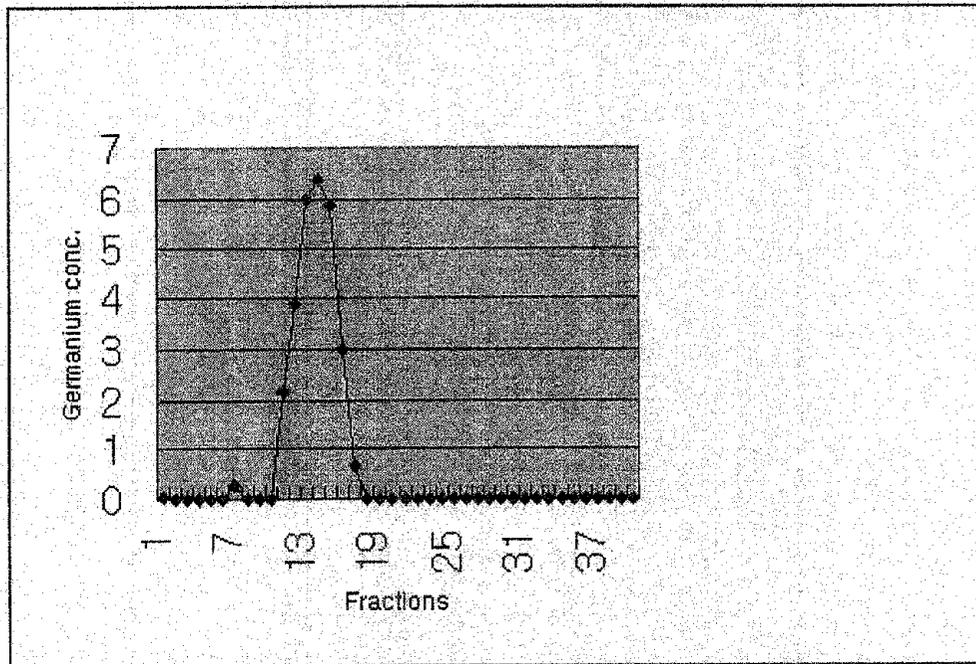


Fig. 8. SDS-PAGE using fractions 13-14 from gel filtration. Lane 1, 2 fractions of ion exchanger; lane 3, 4 protein standard; lane 5, 6 putative protein bound with germanium. Proteins were silver stained.

