

Research Report

Identification and Purification of Dried Yeast-G(Geranti
Bio-Ge, Dried Yeast containing Organic Germanium)

2001. 8. 20.

The National Instrumentation center for Environmental
Management College of Agriculture and Life Sciences,
Seoul National University, Korea

Introduction

Dried Yeast-G('Geranti Bio-Ge' of Geranti Pharm Ltd.) is a bio-product that contains organic-germanium. Formation of Biogermanium was accomplished by adding inorganic form of germanium to the fermented yeast and in turn transforming inorganic germanium into natural organic germanium by interacting with biomolecules including nucleic acid, proteins etc in yeast. Production of Bio-Ge using yeast gains many interests in providing materials for functional food and medicinal product, since organic germanium has been proposed to have many biological functions. The known functions of organic germanium include an anti-cancer activity (Ishida et al, 1979, S. Goodman,UK,1988, J.K.Kang,1994),induction of interferon and activation of NK cells and macrophages(H. Aso,et.al.,F. Suzuki,1985,), lowered blood pressure(The Clinical Rep.,Kiso To Rinsho,1979), against bone-mass decrease in osteoporosis(Asai Res. Ins. 1984), inhibition of tumor growth and metastasis(I.Sato et.al. 1985) anti-mutation activity (Li et. al., 1982, Kada et. al, 1984).

Yeast was known to transform an inorganic germanium into Biogermanium (Klapcinska & Chemielowski, 1986) and also detoxify the inorganic germanium toxic activity by forming bioform (Lynn et al 1997). In present report, we provide solid evidence that 1) inorganic germanium is transformed into Bio-organic-germanium; 2) Biogermanium is a complex with a protein; 3) the complex is isolated and purified.

1. Materials and Methods

1) Material

Sample was dried yeast-G, which Geranti Biopharm company has a patent. Dried yeast-G was 3,275 ppm for germanium activity.

2) Extraction

Freeze-dried yeast-G(Geranti Bio-Ge) was ground under liquid nitrogen and 5 ml of 50 mM Tris-HCl (pH 7.5) buffer was added to the ground powder. After the mixture was centrifuged for 20 min. at 12,000 rpm, the supernatant was obtained and then filtered using 0.45 um filter. The filtrated supernatant is called as "Dried Yeast-G Extract" throughout the report.

3) Determination of Germanium

For determination of germanium activity in sample, ICP instrument (SHIMADZU ICPS-1000) was used. The condition of analysis was 209,49 nm, R.F. Power 1.2kw, and torch height 15mm. All measurements were carried out after calibration using standard solutions (Aldrich).

4) SDS- PAGE Analysis

The purity and molecular weight of Ge-binding protein were determined by SDS-PAGE on a gel prepared with 15% acrylamide by the method of Laemmli. After electrophoresis, gels were subjected to coomassie blue staining.

5) Ion Exchange Chromatography

Liquid chromatography was performed on Liquid Perfusion

Chromatography (Bio-cad, ABI). Ion Exchange Chromatography was carried out on Poros column HQ-20 and Poros HS-20 equilibrated with 50 mM Tris-HCl (pH 7.5) and eluted at a flow rate of 4 ml/min with 50 mM Tris-HCl (pH 7.5) and 1 M NaCl in the same buffer. The filtered extract was injected 2 ml and its elution was monitored by UV-absorption at 280 nm.

6) Gel Filtration

Gel Filtration was carried out on SHODEX Protein column KW-803 (8mm × 300mm) equilibrated with 50 mM Tris-HCl (pH 7.5) and eluted at a flow rate of 0.5 ml/min.

7) Prep. Cell purification

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

8) N-terminal sequencing

N-terminal sequencing was analyzed according to the Edman procedure with an Applied Biosystems 491 sequencer. Prior to sequencing, Ge-binding protein obtained by electrophoresis in 15% polyacryamide gel were electroblotted onto a PVDF membrane (sigma, USA), followed by staining with coomassie brilliant blue.

2. Results

Biogermanium(Geranti Bio-Ge) was isolated and purified as shown in Scheme 1. The results from each step are in below.

1) Ion Exchange Chromatography

Biogermanium from Dried 'Geranti Yeast-G Extract' was shown not to bind to either of anion or cation exchange chromatography as measured by ICP (Figures 1 and 2). Other proteins which do not form a complex with germanium were easily separated from the fractions of Biogermanium. Eluted fractions (#1-3 in Fig. 1) from the cation exchange chromatography, which are flow-through and contain Biogermanium, were pooled and applied to the anion exchange chromatography. The flow-through (#1-3 in Fig. 2) from anion exchange chromatography were collected and then used for the further purifications.

2) Identification of Biogermanium

Fraction # 1-3 from Fig. 2 were further separated using a size exclusion chromatography (Fig. 3). The elution profile and ICP data provide an evidence that fraction # 12-14 contain both of protein and germanium activity, suggesting that these fractions have Ge-binding protein(s). The presence of protein(s) in these fractions are further identified on SDS-PAGE (data not shown). The results from Fig. 3 suggest two possible interpretations.

- A) There are(is) a Ge-binding protein(s) in these fractions.
- B) Under the experimental conditions, inorganic germanium, which does not form a complex with a protein(s), happen to be eluted at these fractions.

To investigate above two possible cases, the following experiments were carried out.

Experiment A) Yeast was grown in the absence of germanium source, i.e. inorganic germanium. The Extract was prepared and purified as described in a section of Materials and Methods and then the results were compared with those from Fig. 3. If Ge-binding protein(s) should present, protein peaks are to elute as shown in Fig. 3 but there would be no peak for germanium.

Experiment B) Yeast was grown under normal conditions with inorganic germanium and the Dried Yeast-G Extract was prepared and purified as described. Just before the Extract was applied to a size exclusion chromatography, inorganic germanium was added to the Extract and then the mixture was loaded to the column. The results from these experiment would provide germanium peaks for Biogermanium and free inorganic germanium.

Experiment C) Inorganic germanium was applied to the size exclusion chromatography to identify the peak position of germanium in the absence of other proteins. Elution profile will be compared with that from B.

Fig. 4 is results from experiment A above. Protein elution profile almost matches with that of Fig. 3 but there are no peaks for germanium activity, suggesting that there is a high chance of the presence of Ge-binding protein(s). Results from Experiment B clearly demonstrate that bound germanium (fraction #12-14) and free germanium (fraction #20-30) are eluted at different positions (Fig. 5). Also inorganic germanium's peaks locate at

#20-30 (Fig. 6). All together we concluded that there is a Ge-binding protein(s) and its elution peaks are easily separated from those of inorganic germanium. Therefore, fraction #12-14 from the size exclusion chromatography must contain Biogermanium.

3) Purification of Biogermanium

Fig. 7 shows that fraction #13-15 from Prep Cell Analysis contain germanium activity. These fractions were freeze-dried followed by SDS-PAGE (Fig. 8). Lane 8 demonstrate putative Ge-binding protein. There is only one protein of which molecular weight is about 34,000 Da. This protein is presumably a Ge-binding protein.

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

Since yeast whole genome was sequenced, N-terminal sequences of the putative Ge-binding protein must provide a clue for the nature of the putative Ge-binding protein. N-terminal sequences for the 34 KDa protein are in Table 1 and the putative Ge-binding protein was named 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 using 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

Using a program BLAST, the N-terminal sequences of GY-45

were matched with NCBI data base of *S. cerevisiae*. 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 are an enzyme enolase and the other two phosphopyruvate hydratase. In particular, the first ten N-terminal sequences of Enolase perfectly matched with those of GY-45. Although there is a difference in molecular weight between GY-45 (34 KDa) and Enolase (47 KDa), the sequence matches can not exclude a possibility that GY-45 might be one of isozymes of Enolase. These speculations are further supported by the observation that metal ions such as Mg and Ca are shown to bind to Enolase as directly found by X-ray crystallography. Currently, we speculate that germanium might replace these metal ions in protein.

3. Conclusions

The above result indicated that the Dried Yeast-G Extract (Geranti Bio-Ge) is bonded with Enolase proteins and biosynthesized organic germanium. N-terminal amino acid sequences using a purified Ge-binding protein have a homology with a metal-binding protein Enolase.

4. Future research

The future research should be concentrated in molecular and biochemical studies to understand the characteristics of Ge-binding protein thoroughly.

References

1. Li AP, Dahl AR, Hill JO(1982) In vitro cytotoxicity and genotoxicity of dichloride and dibutylgermanium dichloride. *Toxicol Appl Pharmacol* 64:482-485
2. Kada T, Mochizuki H, Miyao K(1984) Antimutagenic effects of germanium oxide on Trp-2-induced frameshift mutations in salmonella typhimurium TA98 and TA1538. *Mutat Res* 125: 145-151
3. Ishida N, Suzuki F, Hayashi Y(1979) Antitumor effects of organicgermanium compound(Ge-132) in mouse tumors. *Proc. Of the Japanese Cancer Association Annual meeting*, p193
4. Klapcinska B, Chmielowski J(1986) Binding of germanium to pseudomonas putida cells. *Appl Environ Microbiol* 51:1144-1147
5. Lynn M. Ramsay, Geoffrey M, Gadd.(1997) Mutants of *Sacchomyces cerevisiae* defective in vacuolar function a rolefor the vacuole in toxic metal ion detoxification. *Microbiology Letters* 152:293-298

Scheme 1. Steps in purification of Ge-binding proteins

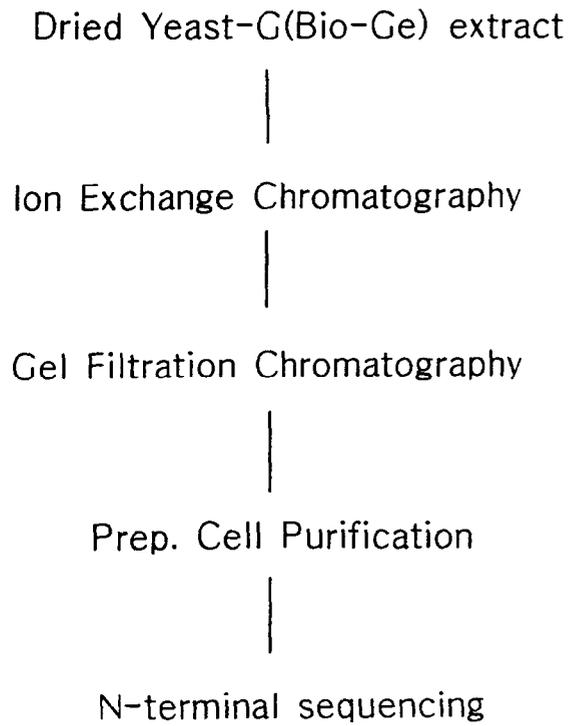


Fig.1. Elution profile from cation ion exchange chromatography for Yeast-G(Bio-Ge) Extract. Fractions with Ge activity were clearly seen in the fraction number 1-3 which were measured using ICP.

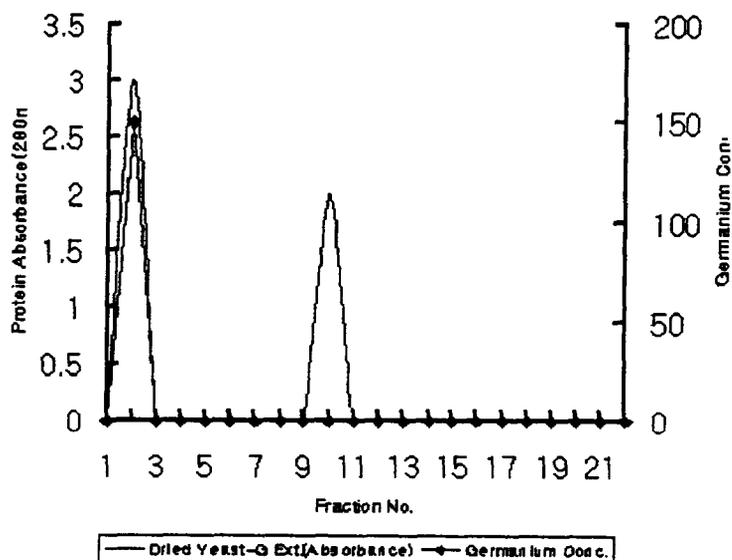


Fig.2. Elution profile from anion ion exchange chromatography for fraction 1-3 in Fig. 1. As shown in Figure 1, fractions with Ge activity(# 1-3) were not bound to the column.

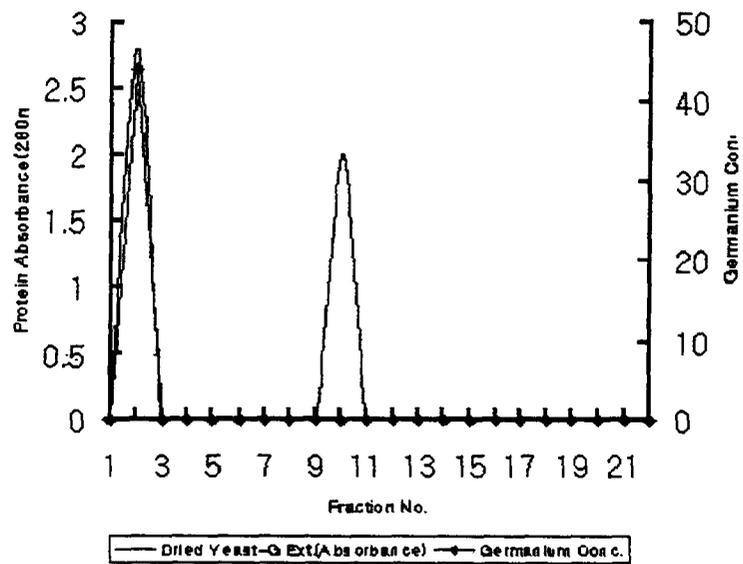


Fig.3. Elution profile of gel filtration (shodex protein KW-803 column) for yeast-G(Bio-Ge) extract. Proteins in fractions 12-14 are expected to be Ge-binding proteins as are indicated with high concentration of Ge.

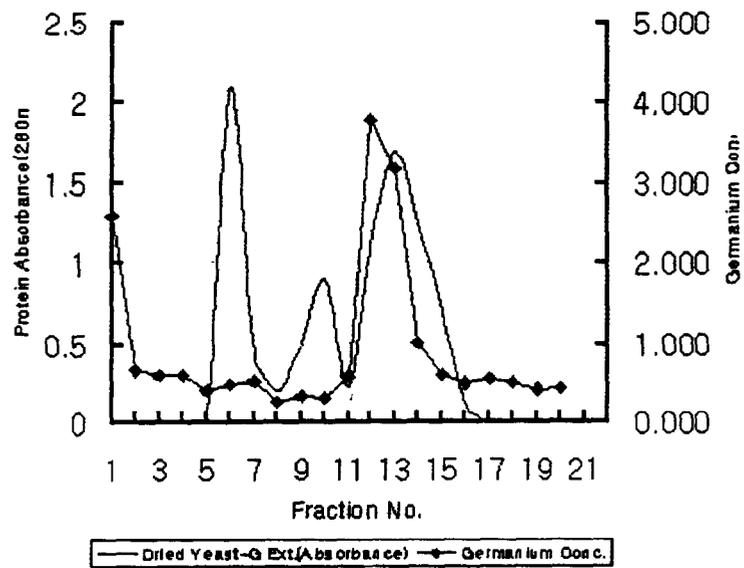


Fig.4. Elution profile of gel filtration (shodex protein KW-803 column) for yeast extract. Yeast were grown in the absence of GeO₂. Unlike Figure 3, there are no trace of Ge for the fractions 12-14.

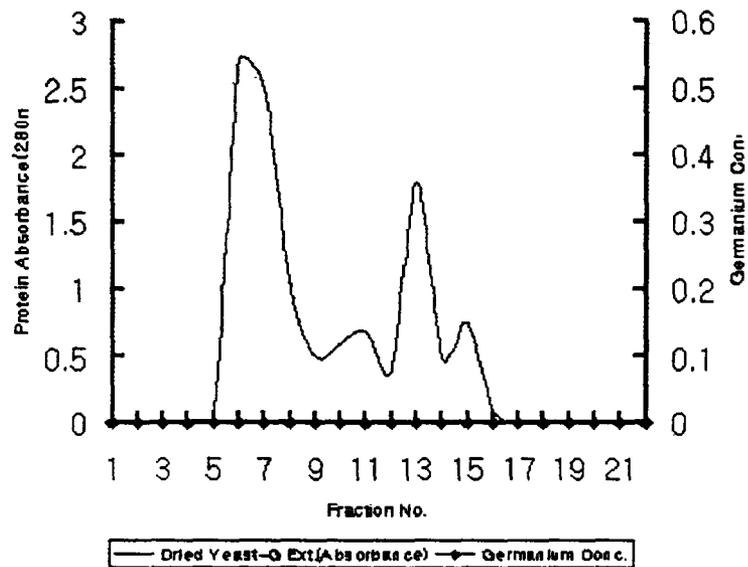


Fig.5. Elution profile of gel filtration (shodex protein KW-803 column) for the mixture of yeast extract and GeO₂. Yeast extract from ion exchange chromatography were mixed with free GeO₂. Due to difference in Ge relative activity between bound form and free form, there are very low concentration of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

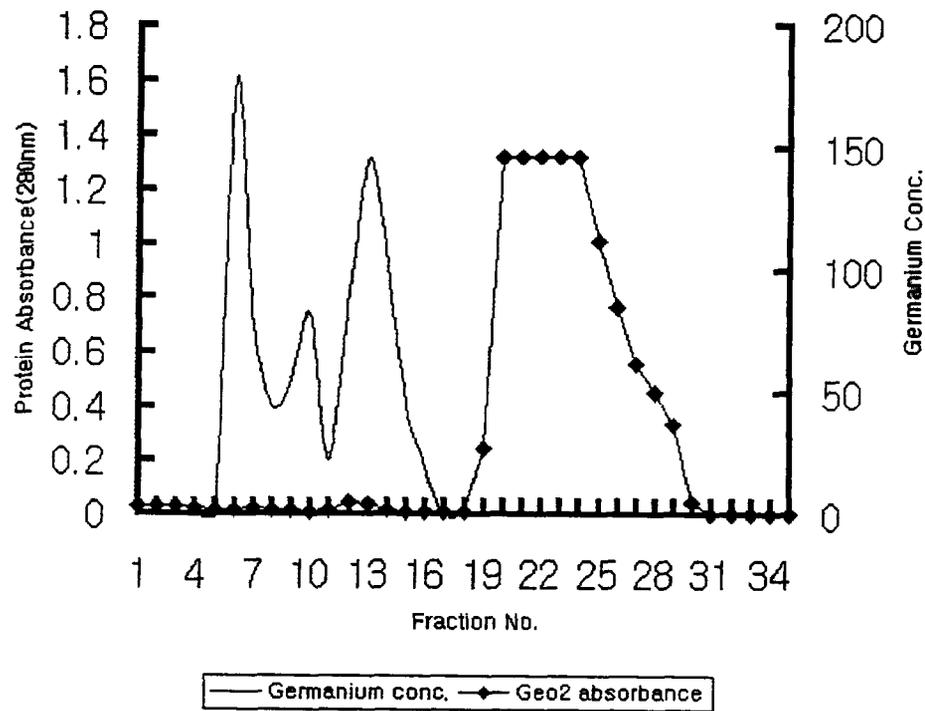


Fig.6. Elution profile of gel filtration (shodex protein KW-803 column) for inorganic GeO₂. Ge peaks are found at the fractions 20-24, suggesting that fractions 13-14 in Figures 3 and 5 are due to the bound form to the proteins.

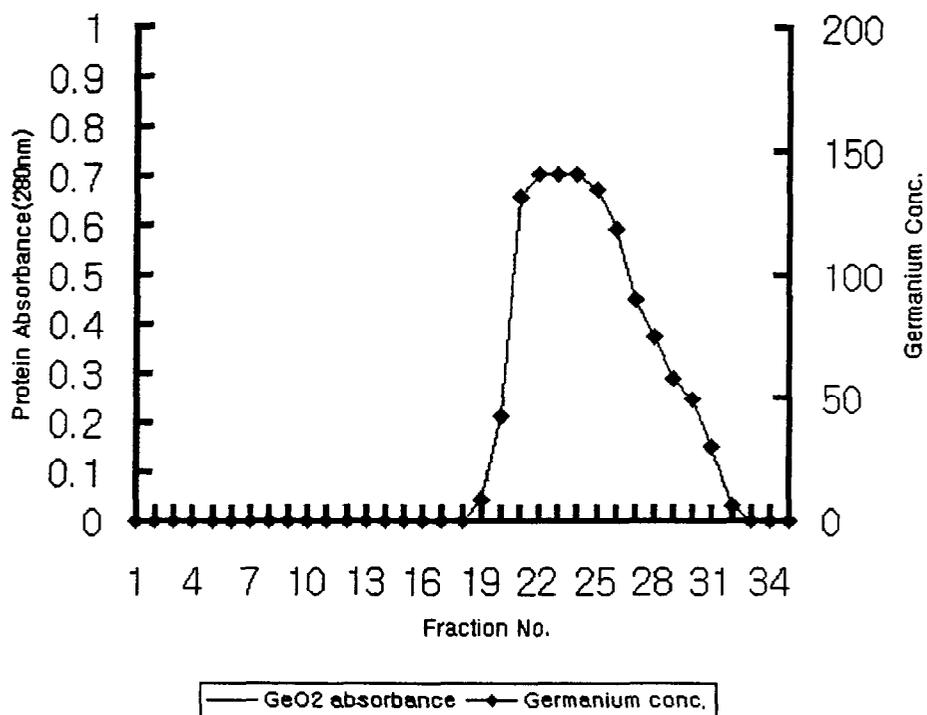


Fig.7. Germanium contents of each fraction separated by prep cell. Fraction with high concentration of Ge correspond to MW of 34 KDa.

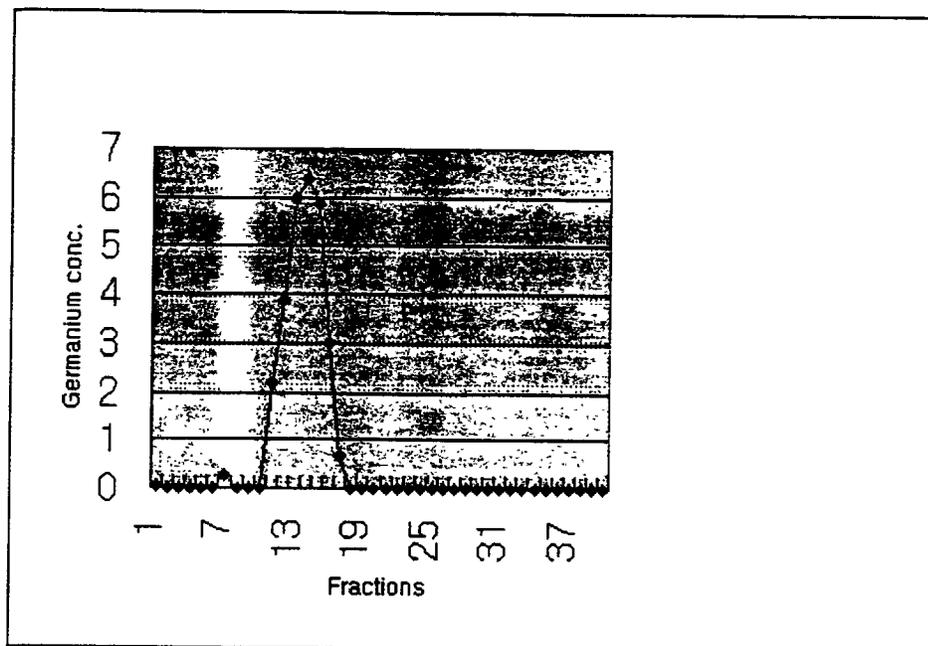


Fig. 8. SDS-PAGE using fractions 13-14 from gel filtration. Lane 1, 2 fractions of ion exchanger; lane 3, 4 protein standard; lane 5, 6 putative protein bound with germanium. Proteins were silver stained.

