

Purification of the Vacuolar Arginine Transporter from *Neurospora crassa*

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*Neurospora crassa*로부터 arginine transporter 의 순수분리

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ABSTRACT: Radioactive N- α -p-nitrobenzoxycarbonyl (NBZ)-L-[2, 3- 3 H] arginyl diazomethane was used as an affinity label for the vacuolar arginine transporter in *Neurospora crassa*. Vacuolar matrix proteins were removed by fracturing the membranes with freeze-thaw method in dry ice/ethanol bath. Vacuolar membrane proteins were then washed with 500 mM NaCl to remove ionically bound derivatives and peripheral membrane proteins from vacuolar membranes. After dissolved in 1% Titon X-100, dissolved vacuolar membrane proteins were separated with molecular sieve column chromatography, anion and cation exchange chromatographies. The arginine transporter was purified giving the purification factor of 1136.

KEY WORDS □ Vacuole, arginine transporter, NBZ arginyl diazomethane.

In *Neurospora crassa*, more than 98% of L-arginine is stored in vacuoles under normal conditions (Weiss, 1976). *In vitro* experiments using vacuolar membrane vesicles made from *N. crassa* showed ATP-dependent and independent arginine transport (Zerez *et al.*, 1986). The best way to find out the importance of the vacuolar arginine compartmentation in arginine metabolism is to compare the vacuolar mutant with wild type. However, since no such mutant is available, it is necessary to make such mutant by genetic engineering. To do this, the arginine transporter protein should be purified, antibody be raised against this, and get the right gene for gene disruption.

One of the problems in attempting to purify the arginine transporter is the possibility that its arginine binding activity might be lost in the presence of detergent. This will cause a big problem in identification of the arginine transporter during and after the

purification process. If the binding activity was lost at anytime, there would be no way to identify the protein after that. Another problem is the small amount of the arginine transporter [less than 0.016% of vacuolar proteins (Paek and Weiss, 1989)]. This makes it necessary to use a sensitive method to detect the arginine transporter.

To solve these problems, radioactive NBZ arginyl diazomethane was synthesized and used to identify the vacuolar arginine transporter (Paek and Weiss, 1989). The binding of this arginyl diazomethane to proteins was stable and could be easily detected because of its radioactivity. Using this arginyl derivative as an affinity label, the arginine transporter was purified from vacuoles labeled with radioactive NBZ arginyl diazomethane, based on differences in charge and size among the different proteins.

MATERIALS AND METHODS

Growth conditions.

Growth conditions for *N. crassa* were as described in a previous paper (Paek and Weiss, 1989).

Vacuole isolation

The procedure for isolation of vacuoles was the same as that described in a previous paper (Paek and Weiss, 1989).

Synthesis of radioactive N- α -p-nitrobenzoxycarbonyl (NBZ) arginyl diazomethane

Radioactive NBZ arginyl diazomethane was synthesized as described in a previous paper (Lee and Weiss, 1989).

Washing of labeled vacuoles with hypotonic buffer and salt.

Vacuoles (100 mg) were labeled with radioactive NBZ arginyl diazomethane (1 μ M, 100 mCi/mmol) in arginine transport assay buffer (25 mM Tris/MES, 1 mM EGTA, pH 7.8) by incubation at 30°C for 10 min. After labeling, vacuoles were collected by centrifugation for 30 min at 10,000 \times g (12,000 rpm) in a SS34 at 4°C. The pellet was dispersed in 1 ml of buffer (1 mM Na₂CO₃, 10 mM Tris/MES, 1 mM EDTA, pH 6.8) and gone through two times of freeze-thaw cycle in a dry-ice/ethanol bath and a 30°C water bath. The mixture was centrifuged for 1 hour at 100,000 \times g in a SW27 rotor. The washed vacuolar pellet was dispersed in buffer (500 mM NaCl, 10 mM MES/Tris, 1 mM EDTA, pH 6.5). The mixture was homogenized with 2-3 strokes at every 15 min for 2 hour while kept in an ice bath. After 2 hour, the mixture was centrifuged at 100,000 \times g in a SW27 rotor. Then proteins were dissolved from salt washed vacuolar membranes with 1% Triton X-100 in buffer (20 mM MES/Tris, 1 mM EDTA, pH 6.5) for 1 hour in an ice bath. Pellets were dispersed with a disposable glass pipette at every 15 min. After incubation, the mixture was centrifuged at 100,000 \times g (24,000 rpm) for 1 hour in a SW27 rotor.

Desalting

Disposable desalting columns were purchased from Bio-Rad. After the column (Bed volume, 3 ml) was equilibrated with elution buffer (25 mM MES/Tris, 1% Triton X-100, 1 mM EDTA, pH

6.5), a 3-ml sample was passed through the column with elution buffer giving 1-ml fractions. The amounts of protein and radioactivity in each fraction were measured as described in a previous paper (Paek and Weiss, 1989).

Cation exchange column chromatography.

Cellex CM (carboxymethyl) cellulose (Bio-Rad) was pretreated according to the instructions by the manufacturer. Seventeen g of resin was suspended in 500 ml of 0.1 M HCl and allowed to swell. After 30 min, the resin was filtered through Whatman filter paper (No. 4) and rinsed with distilled water. It was then suspended in 500 ml of 0.1 M NaOH and allowed to stand for 10 min. The suspension was neutralized with 0.1 M HCl, filtered, and rinsed with equilibrated in 10x concentrated solution of elution buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5). Equilibration was achieved by washing with concentrated buffer until the pH of the supernatant reached the desired pH then followed by washing with elution buffer. After the pH was adjusted, the resin was poured at once into the column (2.8 \times 35 cm). Ten bed volumes of elution buffer containing 1% Triton X-100 were passed through the column. Before each use, the pH of buffer eluted from column was checked with pH paper. Proteins in desalted sample was eluted with elution buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5) collecting sixty 8-ml fractions. After unbound proteins were eluted with 4 bed volumes of elution buffer, proteins bound to the resin were eluted with 1 N NaCl. The column was regenerated by washing with 10 bed volumes of elution buffer.

Anion exchange chromatography.

DEAE anion exchange cellulose (DE-52, prewetted form, Whatman) was swollen in 15 volumes of 10 x concentrated solution of elution buffer (25 mM Tris/MES, 1 mM EDTA, pH 8.0). The treated resin was poured at once into the column (2.8 \times 15 cm) and equilibrated with 4 bed volumes of elution buffer containing 1% Triton X-100. Whenever the elution conditions were changed, 4 bed volumes of new elution buffer were passed through the column and the pH of the buffer eluted from column was checked before the sam-

ple was loaded. Proteins were eluted with elution buffer collecting 4.2-ml fractions. Unbound proteins were eluted with 4 bed volumes of elution buffer containing 1% Triton X-100, and proteins bound to the resin were eluted with 4 bed volumes of 1 N NaCl. The column was regenerated as described for the cation exchange column.

Molecular sieve chromatography.

Proteins in radioactive fractions from anion exchange chromatography were concentrated to 1 ml by ultrafiltration using a Amicon protein concentrator (Amicon, cut mol. wt. 10,000) and eluted on a Sephacryl S-200 Superfine column (Sigma, bed volume, 16 ml) with elution buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5) collecting fifty 4.2-ml fractions.

Protein assay.

The amount of protein was measured with a protein assay kit (Pierce) as described in a previous paper (Lee and Weiss 1989).

RESULTS

Wash with hypotonic buffer.

Since vacuoles contain matrix proteins as well as membrane proteins, it was attempted to remove matrix proteins. As shown in Table 1, a large amount of protein was removed by hypotonic buffer wash. Since vacuoles were expected to be burst open in hypotonic buffer, protein removed in hypotonic buffer should be vacuolar matrix proteins.

Table 1. Hypotonic buffer wash of vacuoles.

Vacuolar proteins (578 μ g) were dispersed in 1 ml of hypotonic buffer (25 mM Tris/MES, 1 mM EGTA, pH 6.8) and collected by centrifugation as described in Materials and Methods. The amount of protein in the supernatant after each wash was measured as described in Materials and Methods.

Hypotonic buffer wash	Protein in Supernatant (μ g)
1st wash	276
2nd wash	40
3rd wash	28
4th wash	24

Salt wash of vacuoles

The arginine transporter is expected to be an integral membrane protein which spans the vacuolar membrane to transport L-arginine rather than a peripheral protein. If the labeled protein were the arginine transporter, it would remain with the vacuolar membrane after washing with high concentration of salt. As shown in Table 2, radioactivity was associated with the vacuolar membrane pellet even after 500 mM NaCl wash. This concentration of salt should be high enough to remove any peripheral proteins as well as destroy any ionic binding between NBZ arginyl diazomethane and proteins. The result suggested that the labeled protein was an integral membrane protein rather than a loosely bound peripheral protein.

Purification of the arginine transporter.

As a first step to purify the arginine transporter (Fig. 1.) which is expected to be a membrane inserted protein, vacuoles were fractured to release matrix proteins. When vacuoles dispersed in hypotonic buffer were frozen in a dry ice/ethanol bath and thawed in a 30°C water bath, large amount of proteins (7 mg from 100 mg vacuolar proteins) were removed from the vacuoles. When vacuolar

Table 2. Salt wash of labeled vacuolar proteins.

Vacuolar proteins (100 μ g) were labelled with radioactive NBZ arginyl diazomethane (0.4 μ M, 50 Ci/mmol) by incubation at 30°C and unreacted derivatives were removed by centrifugation as described in Materials and Methods. Vacuolar pellets were dispersed in 1 ml of various concentrations of NaCl. Vacuoles were collected by centrifugation, and radioactivities in supernatant and pellet were measured as described in Materials and Methods.

Concentration of NaCl (mM)	Radioactivity in	
	Supernatant	Pellet
0	44	3265
25	83	— ^a
50	79	—
100	70	—
250	73	—
500	78	3267

^aRadioactivity was not measured.

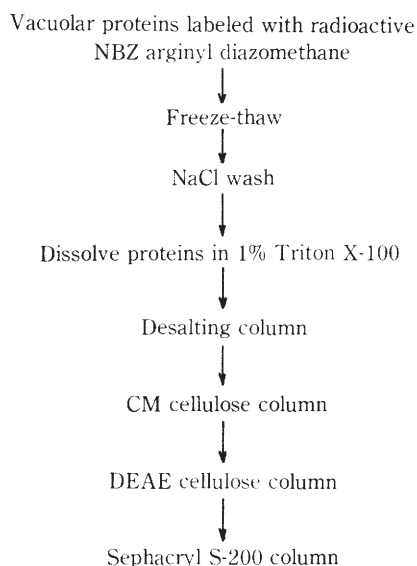


Fig. 1. Purification procedure for the vacuolar arginine transporter in *Neurospora crassa*.

membrane proteins were washed with high concentration of NaCl, radioactivity was remained to the vacuolar membrane pellet even after treatment with 500 mM NaCl (Table 2.) High salt should remove radioactive NBZ arginyl diazomethane attached to proteins by ionic binding and also peripheral membrane proteins. At this step, 9.75 mg of proteins was washed off from vacuolar membrane

Among several non-ionic detergents, Triton X-100 gave the best yield of solubilization (data not shown here). Since it's non-ionic characteristics, it was not necessary to remove the detergent from sample for ion exchange chromatography. For preparation of sample for ion exchange chromatographies, salt was removed from the sample using a desalting column and proteins were eluted with radioactivities in the same fractions (Fig. 2). Since desalting column has a very low cut out molecular weight, it should remove not only salt but also free NBZ arginyl diazomethane which was ionically bound to the protein. No radioactivities were eluted later than proteins suggesting that wash with hypotonic buffer and high concentration of salt remove ionically bound NBZ arginyl diazomethane to proteins.

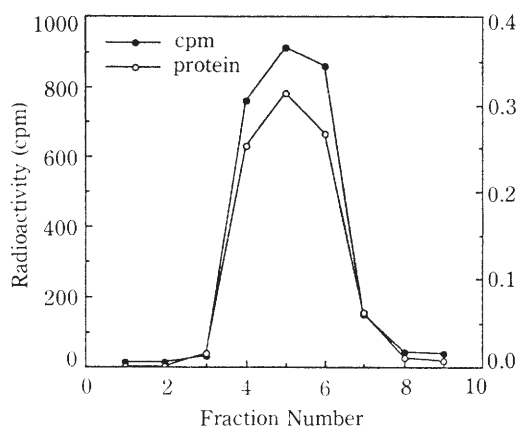


Fig. 2. Desalting column.

Vacuolar membrane proteins labeled with radioactive NBZ arginyl diazomethane washed in hypotonic buffer and high salt were dissolved in 1% Triton X-100 as described in Materials and Methods. Dissolved proteins were chromatographed on a desalting column (bed volume, 3 ml) with buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5) collecting ten 1-ml fractions. Radioactivity and the amount of protein in 10 μ l of each fraction were measured as described in Materials and Methods.

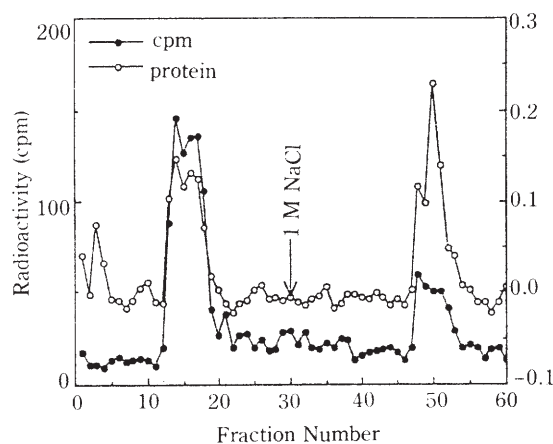


Fig. 3. CM cellulose cation exchange chromatography.

Fractions containing radioactivity from the desalting column in Fig. 2 were loaded on a CM cellulose column (bed volume, 100 ml). Proteins were eluted with buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5) collecting sixty 8 ml fractions. NaCl (1 M) was added where indicated. Radioactivity and the amount of protein in 100 μ l of each fraction were measured as described in Materials and Methods.

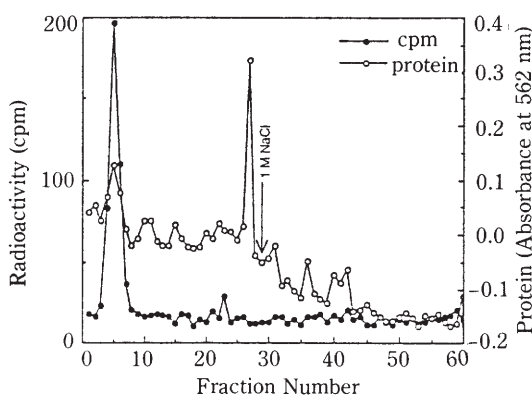


Fig. 4. DEAE cellulose anion exchange chromatography.

Fractions containing radioactivity from the CM cellulose column in Fig. 3 were concentrated to 3 ml and then chromatographed on a DEAE cellulose column (bed volume, 35 ml) with elution buffer (1% Triton X-100, 25 mM Tris/MES, 1 mM EDTA, pH 8.0). NaCl (1 M) was added where indicated. Sixty 4-ml fractions were collected; radioactivity and the amount of protein in 50 μ l of each fraction were measured as described in Materials and Methods.

Two successive ion exchange column chromatographies separated the arginine transporter from most of vacuolar membrane proteins [95% by a cation exchange column (Fig. 3) and 94% of the remaining proteins by an anion exchange column (Fig. 4)]. When the fractions containing radioactively labeled proteins from the anion exchange column were eluted on a Sephacryl S-200 Superfine column (Fig. 5), the arginine transporter was further purified. Since the amount of arginine transporter in vacuoles was calculated to represent 0.016% of the vacuolar proteins (Paek and Weiss, 1989), 16 μ g was expected for a 100% yield from 100 mg of vacuolar proteins. Radioactive fractions eluted from a Sephacryl S-200 Superfine column had 2 μ g of protein, giving a yield of 12%. The yield of radioactivity was only 3%. This may result from the loss of label from the protein at the basic pH used for anion exchange chromatography. The specific radioactivity increased from 5 cpm/mg to 5682 cpm/mg, giving a purification factor of 1136 (Table 3). It seems clear that the radioactively labeled arginine transporter protein can be purified using a combination of ion exchange chromatog-

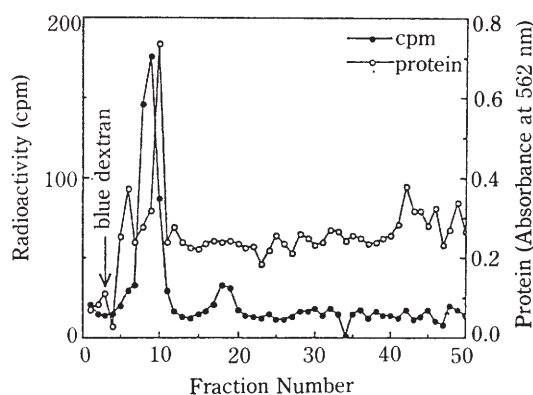


Fig. 5. Molecular sieve column chromatography.

Fractions containing radioactivity from the DEAE cellulose column in Fig. 4 were concentrated (1 ml) and then chromatographed on a Sephacryl S-200 Superfine (bed volume, 16 ml) with elution buffer (1% Triton X-100, 25 mM MES/Tris, 1 mM EDTA, pH 6.5) collecting fifty 4.2-ml fractions. Radioactivity and the amount of protein in 100 μ l of each fraction were measured as described in Materials and Methods.

Table 3. Purification of the arginine carrier using ion exchange and molecular sieve chromatography.

Purification Step	Protein (μ g)	Radioactivity (cpm)	Specific Radioactivity (cpm/ μ g)
Desalting	81,291	421,420	5
CM Cellulose	3,956	234,752	59
DEAE Cellulose	256	94,400	368
Sephacryl S-200	2	13,524	5682

raphy, molecular sieve chromatography.

DISCUSSION

Purification of the vacuolar arginine transporter was achieved using techniques based on the charge and size of proteins. Proteins which fail to bind to CM cellulose must be neutral or anionic, and anionic proteins will bind to DEAE cellulose. These results suggested that the arginine transporter has no charge on it and/or has charged groups which have pKa's around pH 6.5 and 8.0. This was expected for the membrane inserted protein which is mostly composed of uncharged amino acids.

Two good candidates for charged groups on vacuolar arginine transporter are imidazolium of histidine (pKa 5.6-7.0) and the sulfhydryl group of cysteine [pKa 8.0-9.0, (Smith *et al.*, 1983)]. These two amino acids have side chains which can be easily attached to NBZ arginyl diazomethane via ketene group. Especially sulfhydryl group is the most likely binding site of ketene group. In a previous work (Lee and Weiss, 1989), cysteinyl blocking reagents inhibited labeling and -SH is stronger nucleophile than -NH. Cysteine has been suggested to be involved in the transport mechanism for basic amino acids in yeast vacuoles (Ohsumi and Anraku, 1981). To identify the binding site on the arginine transporter for NBZ arginyl diazomethane more work must be done. One approach would be amino acid analysis of the labeled protein and determine which amino acid has the derivative attached to it.

In addition, more than 15% of total vacuolar proteins were removed by freeze-thaw cycle and salt wash, while radioactivity was remained with vacuolar membrane. This suggested that the labeled protein was not a periplasmic binding protein for recognition like the arginine transporter in *Escherichia coli* (Striessing *et al.*, 1986) or not a matrix protein. In *E. coli*, arginine transport through the plasma membrane is accomplished via shock-sensitive transport system which requires both membrane-bound components and periplasmic binding proteins for recognition of the substrate. In vacuoles of *N. crassa*, the arginine transporter may carry both functions, recognition and transport of L-arginine.

Since the arginine transporter has been identified and its purification has been achieved, it can be purified on a large scale (more than 4 μ g) for amino acid sequencing. Oligopeptides could be synthesized based on the amino acid sequence, and antibodies could be obtained. With the antibodies, the synthesis and transport of the arginine transporter protein into the vacuolar membrane will be studied. The gene for the vacuolar arginine transporter could be isolated using the antibodies or oligonucleotides synthesized based on the amino acid sequence. Using genetic engineering techniques, gene replacement could be tried with a mutated gene. The newly produced mutant could be compared with a wild-type strain by genetic, physiologic, biochemical, and biological tools to find out the importance of arginine compartmentation in arginine metabolism. NBZ arginyl diazomethane can be used to screen possible vacuolar mutants telling us how many arginine transporter exist on vacuole or whether the arginine recognition site has been altered.

Furthermore, NBZ arginyl diazomethane might be used to label the feedback inhibitable enzyme, acetyl glutamate synthase in the inner mitochondrial membrane which has the same specificity for arginine recognition, L-configuration and an intact guanido side chain.

Vacuolar transporters for L-lysine and L-ornithine, which are sequestered in vacuoles of *N. crassa* (Vaughn and Davis, 1981), might be identified and purified using diazo derivatives of these amino acids following the same procedures used for isolation of the vacuolar arginine transporter.

적 요

Radioactive NBZ arginyl diazomethane 으로 액포를 표지 한 뒤에, 액포내의 단백질과 세포막 겉에 존재하는 단백질을 각각 저농도 완충용액과 높은 농도의 염용액으로 제거시켰다. 액포막 단백질을 Triton X-100 으로 녹인 후, molecular sieve column chromatography 와 ion exchange column chromatography 를 사용하여 arginine transporter 를 분리하였다.

REFERENCES

1. Lee, Y. and Weiss, R.L. (1989) Affinity labeling of the vacuolar arginine transporter in *Neurospora crassa*. The Korean Journal of Microbiol. In this volume.
2. Ohsumi, Y. and Anraku, Y. (1981) Active transport of basic amino acids driven by a proton

- motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **256**, 2078-2082.
3. Paek, Y.L. and Weiss, R.L. (1989) Identification of an arginine transporter in the vacuolar membrane of *Neurospora crassa*. *J. Biol. Chem.* **264**, 7285-7290
4. Smith, E.L., Hill, R.L., Lehman, I.R., Lefkowitz, R.J., Handler, P. and White, A.W. (1983) Principles of Biochemistry, pp32, McGraw-Hill, Inc. New York.
5. Striessing, J., Moosburger, K., Goll, A. and Ferry, D.R., Glossmann, H. (1986) Stereoselective photoaffinity labeling of the purified 1, 4-dihydropyridine receptor of the voltage-dependent calcium channel. *Eur. J. Biochem.* **161**, 603-609.
6. Vaughn, L.E. and Davis, R.H. (1981) Purification of vacuoles from *Neurospora crassa*. *Molec. Cellul. Biol.* **1**, 797-806.
7. Weiss, R.L. (1976) Compartmentation and control of arginine metabolism in *Neurospora*, *J. Bacteriol.* **126**, 1173-1179.
8. Zerez, C.R., Weiss, R.L., Franklin, C.F. and Bowman, B.J. (1986) The properties of arginine transport in vacuolar membrane vesicles of *Neurospora crassa*. *J. Biol. Chem.* **261**, 8877-8882.

(Received May 24, 1989)