Activity and Inhibitory study of Reconstituted Lyp1 from *Saccharomyces cerevisiae*

Bachelor Research Project

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Abstract

Lyp1 is a lysine permease from the SLC12A transporter family, which is also a unidirectional secondary transporter from *Saccharomyces cerevisiae*. It has 12 trans-membrane spanning segments with both N- and C-terminus inside the plasma membrane. Lyp1 equipped with a GFP tag was heterologously expressed in *Pichia pastoris* with methanol as the induction agent. ^{14}C-lysine uptake assay is the main reconstituted Lyp1 activity and inhibitory measurement. Since the exact topological three-dimensional structure of Lyp1 has not been revealed yet, we purified and reconstituted Lyp1 into liposomes containing different ergosterol mole percentages; among three ergosterol percentages, 10 mol percent achieved the highest activity. Because of the different affinity of the two sides of Lyp1, this lysine-specific permease preferably transports lysine from outside of the cell to inside. As possible inhibitors, lysine and arginine analogues, antidepressants and natamycin were tested at 20 to million-fold excess to lysine. By measuring the counts of radio activit, we observed full inhibition with a high concentration of 100 mM L-ornithine but also lower concentration of 20 μM and 200 μM thialysine. We then found the relative potential structural stabilizer of Lyp1.

Introduction

Yeast amino acid transporters (YATs), which locate across most of the plasma membranes present in yeast cells, are the one of the cell synthetic nitrogen sources. [1] The addition of natural amino acids or their analogues during the cell growth can increase the sorting of Amino acid permeases (AAPs) to the vacuole. [2] General amino acid permease (Gap1) have more preferably machinery collection on amino acid-like compounds than a particular nitrogen source, this indicates amino acid analgoues could be a competitive transport agent.[11] Lyp1 is a proton-dependent lysine permease from the amino-polyamine-organocation (APC) superfamily, a superfamily that comprises secondary active transporters. In animals, especially mammalians, cation-coupled cotransporters play an essential role in cell volume regulation, ion concentration modulation and blood pressure control.[10] The association with renal diseases such as hypokalemia, metabolic alkalosis and hypercalciuria, has been documented for neutral cation-coupled cotransporters (NKCC1)[10]. The general model of AAPs has 12 trans-membrane segments with both N- and C-terminus inside cell plasma membrane. There is not much evidence that Lyp1 has affinity for other aa's is low, but exclusivity binding for L-lysine.[3] However, the three dimensional topological structure of this lysine-specific permease has not been solved yet. Previous studies concluded that Gap1 and arginine specific permease (Can1) have low dependency on ubiquitylation.[4] This prevents them from being affected by attaching ubiquitin, which is another target protein signaling and catalyzing the molecule degradation.

The transport of lysine is driven by proton motive force (PMF), coupled with proton influx, Lyp1 acts as a symporter across the plasma membrane to take up lysine from extracellular matrix with higher concentration. During the transportation of positively charged lysine, the membrane potential will increase. Asymmetric lysine transport was revealed by significant high affinity from out-to-in than in-to-out ($K_M^{in \to out}$), resulting in a clear directional lysine moving flow.[2] Overall, ^{14}C-lysine uptake assay was selected to be the optimal method of measuring Lyp1 activity.

The activity measurements start from *in vivo* with the expression of Lyp1 in *P. pastoris,* a fast eukaryotic expression system sharing genetic similarity with *Saccharomyces*, which can generally provide high expression levels heterologously. To achieve an optimal expression of Lyp1 in *P.pastoris*, the solution is to induce methanol to the incubation system. When the yeast cells are grown relied on an only carbon source, methanol, the AOX enzyme can reach 30% of the total cell protein.[5] Under the control of *AXO1* promotor, alcohol oxidase inside *P. pastoris* is encoded. With the accumulation of lysine inside the plasma membrane, the activity of Lyp1 was detected through radio-active uptake assay after the purification and reconstitution to lipids. In this study, we confirmed the localization of expressed Lyp1 in plasma membrane. The removal of Lyp1 from plasma membrane, which intracellular metabolism will be influenced by its sequestion of solutes in the organelles, result in diminishing the activity of Lyp1, so the *in vivo* mechanistic studies were hindered.[2] To avoid this fact, we reconstituted Lyp1 transporters to vesicle lipids with different ergosterol mole percentage after purification with Ni^{2+}-sepharose. In comparison with cholesterol, structurally, ergosterol has two more carbon-carbon double bonds and one methyl group. It can be concluded from the composition studies that unsaturation of acyl chains and negatively charged lipids are ideal selections for stimulation on Lyp1 activities.[2] Furthermore, inhibition of the protein activity was examined by applying antidepressant, lysine and arginine analogues, antifungal drug and vescular transporter bloker. Natamycin was also introduced in the first uptake assay, which is an antifungal medication aiming at destroy the fungal cell membrane by special binding to ergosterol. We observed the one-way transport motered by proton motive force, and discovered that S-aminoethyl-L-cysteine (thialysine) is a potent inhibitor of Lyp1.

Results

Expression and purification of Lyp1 in Pichia *cells*

To ensure Lysine permease from *Saccharomyces cerevisiae* is correctly expressed in the cell membrane *Picha pastoris*, transporters equipped with GFP (Green Fluorescent Protein) tag at C-terminus allows the visual localization of the Lyp1. According to previous studies, there is not much distinguishable transport activity difference with or without the fluorescence tag on Lyp1 while expressing the protein heterologously. Also, the highest expression level of Lyp1 was achieved in *P. pastoris*.[2]

Our selection for a culture medium was depended on the fluorescence intensity and uniformity of *P. pastoris* membrane. Yeast cells from both buffered Mannitol-complex Medium (BMMY) and Minimal Mannitol Yeast Histidine (MMYH) were confocally imaged after 37 and 53 incubation hours. Cells in the minimal medium have more intense fluorescence signals on the plasma membrane than those in the complex medium (Fig 1. C and D). The final OD_{600} of BMMY and MMYH are 9.4 and 11.6 after 48 hours of incubation. Even though inevitable clustered cells appeared during the imaging screening, apparent preference on the minimal medium can be determined. The in-gel fluorescence result (Fig1. B) showed that clear bands appeared above the ladder noted as 70 kDa for both cultures. This indicated the existence of Lyp1 during incubation. However, some degradation of Lyp1 protein appeared in the minimal culture as there are pretty much bands between 70 and 20 kDa. The degradation of the protien happened after treating under room temperature for 48 hours, even though, the fluorescence bands of mininal media are still clear enough to identify. After the *P. pastoris* plasma membrane was extracted from preculture, Lyp1 purification was conducted according to its Ni^{2+} affinity, followed by size-exclusion chromatography (Fig1. A). There will be inactivated transporters due to filtration during reconstitution and dual orientation[6]. This can result in less functional transporters than anticipated.

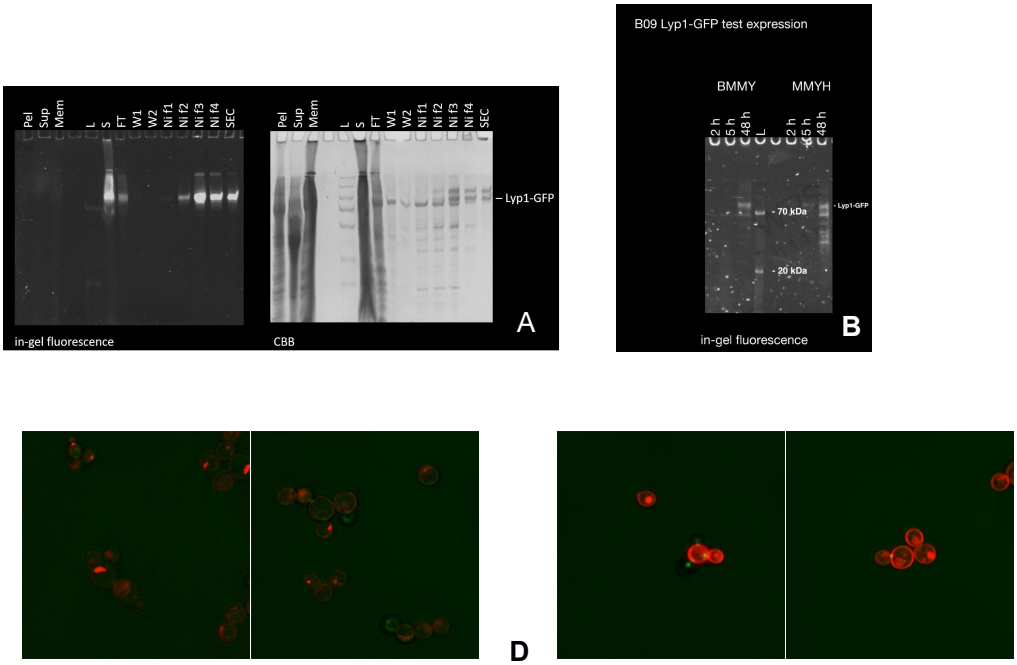


Figure 1: Fluorescence images of Lyp1 expression in *Pichia p.* and purification gel results. (A) The purification results of fluorescence image of SDS-Page gel. The double band shown in Coomassie Blue staining right aside

Lyp1-GFP could possibly be unfolded or aggregated GFP tag which is suggested by the corresponded fluorescence image on the left. There is only one clear fluorescent band of size-exclusion chromatography product. (B) In-gel fluorescence image after incubation for 2, 5 and 48 hours with BMMY and MMYH, respectively. Compared with figure (A), (B) was conducted in a way bigger (six liter) culture. (C), (D) Confocal microscopy image of *Pichia p.* cells growth after 27 and 53 hour in BMMY and MMYH, respectively.

Kinetics of lysine transport

Since the driving force of Lyp1 transport is the influx of protons, it is necessary to create membrane potential (Δψ) by diluting proteoliposomes that contained potassium-acetate into sodium-phospate rich solution. Membrane potential calculation formula is given as:

$$\Delta\Psi = -Z\log\frac{[K^+]_{in}}{[K^+]_{out}}$$

This is equivalent to the minus of pH gradient. Valinomycin, which acts as an antibiotic potassium transporter, was added to the outside solution to ensure the outward potassium ion diffusion, which can build up membrane potential, mainly contributing to the transport of lysine through Lyp1. The driving force generated by the pH gradient (ZΔpH), which is because the diffusion of acetic acid, can be calculated through the formula derived from the Nernst equation, given as:

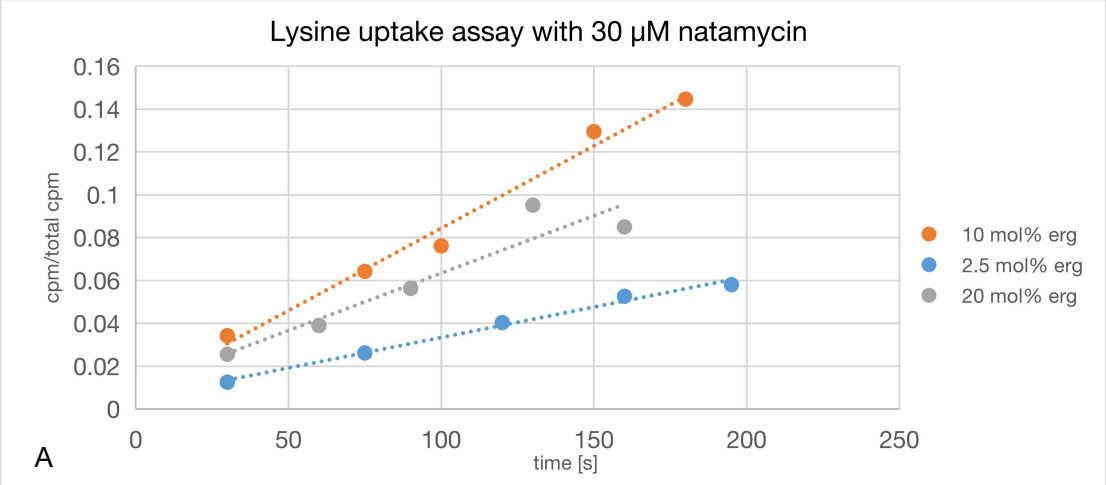
$$Z\Delta pH = \sim Z\log\frac{[acetate]_{in}}{[acetate]_{out}}$$

, where Z = 2.3RT/F, R is gas constant and F is Faraday constant. The concentration ratio of acetic acid inside to outside directly equals the dilution factor of proteoliposomes in the outside buffer. The ratio of the inside acetate over outside ones is exactly the same as the So, ZΔpH in the 14C-lysine uptake assay is -87.4 mV as calculated.Proton motive force (PMF) here was calculated as 174.8 mV (PMF = Δψ-ZΔpH), where the sum of contributions of pH gradient and membrane potential.

Dependency of lipid composition on Lyp1 activity

In the 14C-lysine uptake assay, the activity of Lyp1 transport can depend on three different lipid compositions. The lipids consisted of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and ergosterol. The first two are geometrically column-shaped, which means they are bilayer lipids and are mildly affected by osmotic pressure and membrane curvature. POPE is a non-bilayer lipid with no net charge; Lyp1 was reported to have active in the absence of this lipid but saturation was observed at approximately 10 mole percent. [2] According to Table 1, we altered the lipid composition of

ergosterol to achieve the best activity of Lyp1 in proteoliposomes. The three selected mole percentage were based on Lyp1 turnover number as a function of ergosterol composition [7]. 2.5 mol% was selected as the representative of the increasing period of Lyp1 activity, while 10 mol% and 20 mol% were picked in the stationary stage of that. Usually, ergosterol occurs at approximately 30 mol% in the plasma membrane of yeast cells.[2] In previous studies, the more ergosterol present in constructed lipids, the more active Lyp1 was, increasing up to 10 mol%.Also, the results of 14C-lysine uptake assay showed that 10 mol% of ergosterol present in the lipids gave the highest counts per minute (cpm) ratio regardless of 30 μM natamycin. In comparison with the samples with or without the presence of natamycin, mild activity inhibitory was shown (Fig. 2A, 2B). The addition of this fungi resistant to proteoliposome is to inhibit the activity of Lyp1 as a control sample.



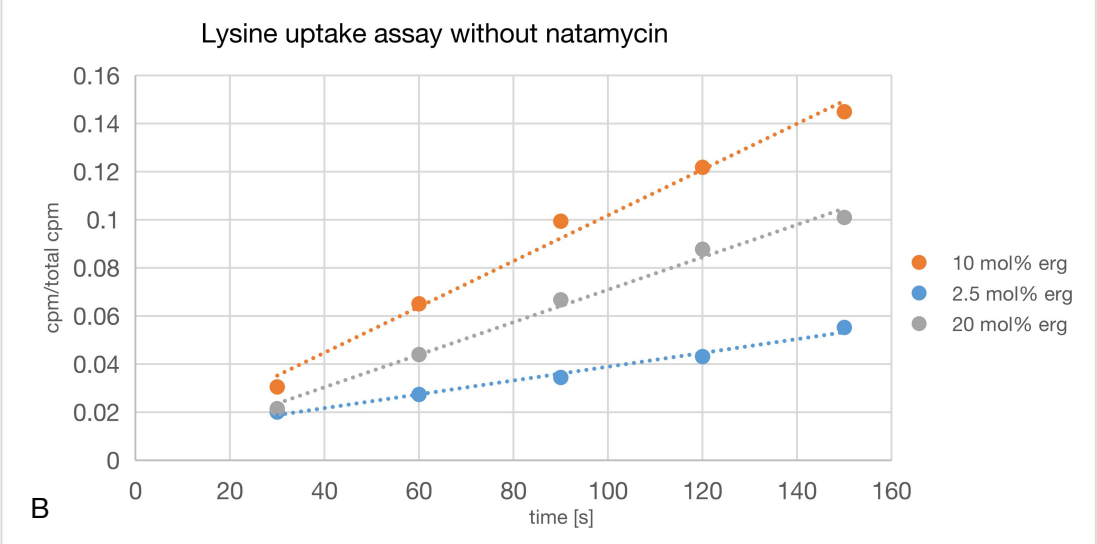
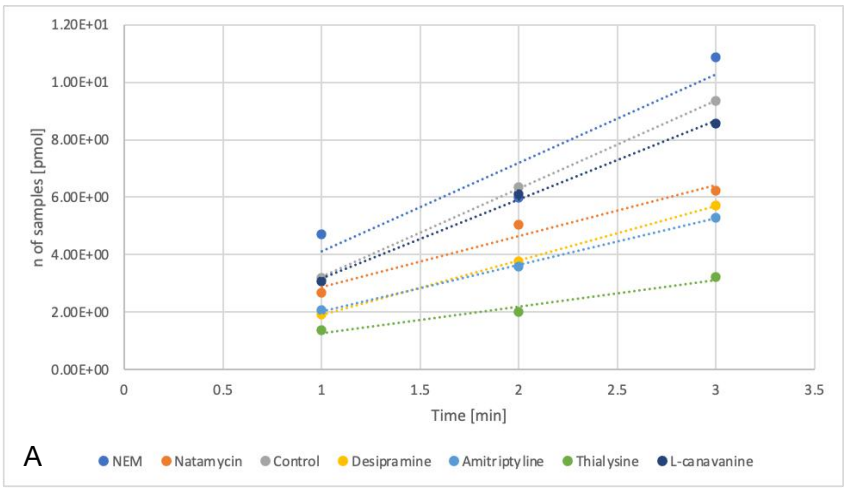


Figure 2. Lyp1 activity versus ergosterol composition. (A) Lysine uptake of Lyp1 without natamycin. (B) Lysine

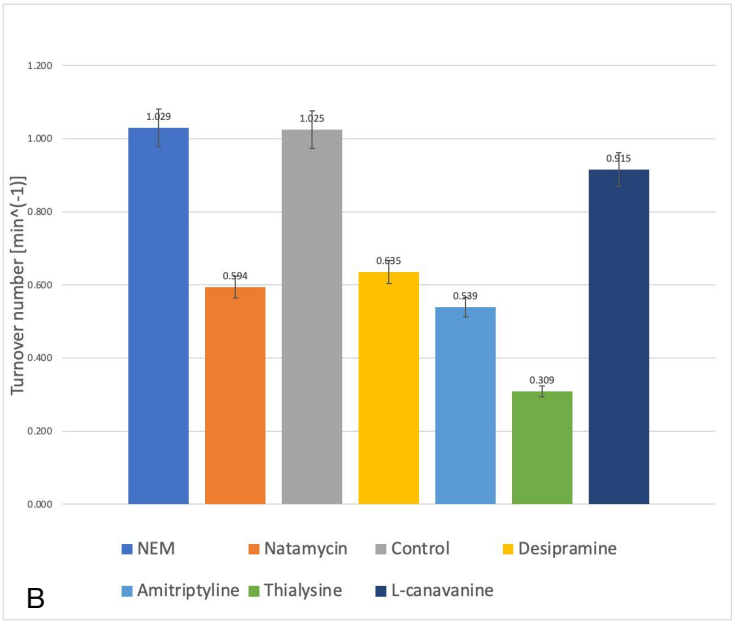
uptake of lysine with 30 μM natamycin. The dash line shown in the figures are linear trendline of counts per minutes versus time.

Inhibitory assay of lysine transport

On purpose of exploring the activity of reconstituted Lyp1 protein, different inhibitors were introduced in the uptake assay.



A



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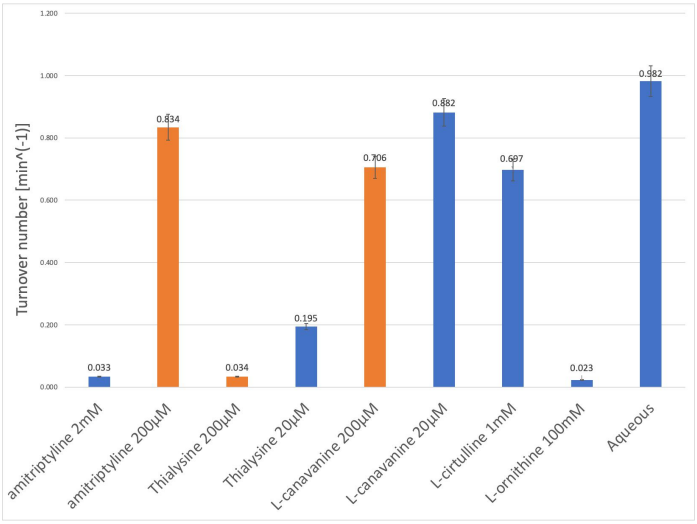

C

Figure 3: Kinetic lysine transport results (A) The amount of lysine transported through reconstituted Lyp1 with the presence of each inhibitor versus time. The slope here indicated the transportation speed of lysine (Table 3 in the appendix). (B) Lysine transport of membrane reconstituted Lyp1 with the presence of inhibitors and natamycin. (C) Lysine transport of membrane reconstituted Lyp1 with the presence of inhibitors without time interval sample taking in 20 mol% ergosterol lipids. The inhibitors used here are considered to have stronger inhibitory than that of (B). The orange bar in (C) indicates the same end concentration. The turnover number is calculated under the assumption that all reconstituted transporters were functional. Only in (C), all fits and errors are derived from three independent experiments. Error bars represent standard deviation.

The (Fig. 3C) compounds used in the assay can be roughly classified as competitive and non-competitive inhibitors. After extrusion, freezing and thaw cycles, the orientation of reconstituted Lyp1 was randomized in the vesicles. Generally, inhibition of lysine transport can be conducted by its analogues, such as some other positively charged amino acids, for example arginine and ornithine. In previous *in vivo* studies, the uptake inhibition of lysine is less than 50% of the total uptake with 100 mM histidine or ornithine (2000-fold excess over lysine)[1]. S-aminoethyl-L-cysteine (thialysine), another lysine analogue, was reported to have competitive inhibition on LysP, which is a bacterial lysine-specific transporter from *Salmonella typhimurium*.[8] Moreover, the inhibitor of general amino acid permease (Gap1) and arginine amino acid permease (Can1), L-citrulline was tested in the assay at 1 mM according to reference [9]. With further research on general inhibitors, amitriptyline and desipramine were chosen as potential non-competitive inhibitors, since these two antidepressants are capable to block non-selectively many receptors and transporters according to tetracyclic antidepressant binding profiles. [13]

Since L-ornithine was added as the hydrochloride salt, the presence of this inhibitor at 100 mM could potentially change the pH value of the outside buffer, and also its osmotic pressure. This could have resulted in denaturation and dysfunction of Lyp1. Even though the radio-active lysine uptake results showed potent inhibition of 100 mM L-ornithine present in the solution, the mechanism might not be simple substrate-binding inhibition.

Thialysine would further be selected as Lyp1 stabilizer and its corresponded inhibition reached 80.1% and 96.5% with 20- and 200-folds, respectively. These results also implied that complete inhibition could be achieved without denaturating Lyp1. The pKa of thialysine is 9.82 [12], which is smaller than that of lysine, which is slightly more difficult to be protonated at pH 6.0. This indicates that if the pH of the buffer system is slightly higher, then the inhibitory of thialysine could be lower than the current.

Conclusion

Our study repeated and expanded the work of van't Klooster *et al*.[2, 7] who has pointed out the ergosterol-dependence of Lyp1 from *S. cerevisiae*. Lyp1 was heterologously expressed in *P. pastoris* with the induction of methanol. Given that Lyp1 is a relatively slow symporter of lysine coupled with the influx of proton, the ^{14}C-lysine uptake assay was designed based on membrane potential and pH gradients created by potassium ion and acetic acid concentration difference from two sides of the transporter. We have shown that a 20- to 200-fold excess of thialysine can reach more than 80 percent inhibition. Therefore, thialysine can potentially be used to stabilize Lyp1 during purification, or as a negative control in uptake experiments. The exclusivity of Lyp1 was indirectly proved by low inhibitory of arginine analogues, L-canavanine and L-citrulline, which were applied more than 1000-fold according to the lysine concentration. We tested the effect of two antidepressants on the transport efficiency, both amitriptyline and desipramine showed some diminishment of Lyp1 activity at 200-fold the lysine concentration. We have also demonstrated that reconstituted Lyp1 activity has less dependency of ergosterol mole percentage once it has exceeded 10 mol%. Reconstitution can be further applied to nanodiscs with purified MSP, allowing to determine the three-dimensional topological structure of Lyp1 in a native-like lipid environment.

Method and Measurements

Lyp1 (over) expression system in P. pastoris

Lyp1-GFP-his_6 *P. pastoris* were grown in Buffered Glycerol-complex (BGMY) containing medium 1% (w/v) glycerol, 1% (w/v) yeast extract, 2% peptone, 100 mM potassium phosphate at pH 6.0, 1.34% (w/v) Yeast Nitrogen Base (YNB) without amino acid after filtration, 5-10% (w/v) biotin or Minimal Glycerol Yeast Histidine (MGYH) with 1% (w/v) glycerol, 2% (v/w) D-Mannitol, 1.34% (w/v) YNB without amino acid, 0.04‰ L-histidine, 0.0004‰ biotin at 30°C and approximately 200 rpm overnight. The carbon source was changed to D-Mannitol by dilution of precultured cells. Overnight growth led to an OD_{600} around 1.0 in 1.5 liter culture in Minimal Mannitol Medium. After the incubation with D-Mannitol as a carbon source, methanol was added to 0.5% (V/V). The cells were then cooled on ice and centrifuged at 7500 × *g* for 15 minutes using JLA9.100 rotor. After washing the cell with precooled Cell Resuspension/Breaking Buffer (CRB) containing 50 mM Tris-HCl pH6.7, 1 mM EDTA, 0.6 M sorbitol, pellets were centrifuged again with the same speed and time at 4°C. Harvesting cells were completed by resuspension to an OD_{600} of 200 and addition of 1mM PMSF and one protease inhibitor tablet (complete EDTA-free™, ROCHE) per 100 mL.

Extraction and purification of plasma membrane

Cells were broken by Maximator cell disrupter four sequential passes at about 40 kpsi. After the last passage of breakup, phenylmethanesulfonylfluoride (PMSF) was added to the mixture to the final concentration of 1 mM as well as another protease inhibitor tablet. Intact cells and large debris were removed by centrifugation at 18,000 × g for 30 minutes at 4°C. Empty 45 TI rotor tubes need to be weighted before loading the supernatant. The membrane fraction was collected after ultra-centrifugation at 186,000 × *g* for 2 hours at 4°C. Membrane pellets were mixed to homogeneity by means of Potter-Elvehjem tissue grinder in membrane resuspension buffer (0,1mM $CaCl_2$, 0.3 M sucrose, 20 mM Tris-HCl (pH7.5)). 1 mM PMSF, 1 µM Pepstatin A and 1 tablet of protease inhibitor were added to the membrane mixture. The membranes were suspended to about 400 mg/ml (wet weight). Then the membrane were aliquoted in 1 mL volumes. After quick freeze in liquid nitrogen, the samples were stored at -80°C.

Lyp1 protein purification

The further purification of Lyp1 from microsomal membranes was conducted by solubilization in buffer (0.05 M NaCl, 10% Glycerol, 1% n-dodecyl-β-maltoside, 0.05 M ammonium acetate, 0.015 M imidazole at pH7.5, 2 mM PMSF). Then ultracentrifugation (rotor type MLA-80) at 80,000 rpm for 20 minutes at 4°C. Meanwhile, Ni^{2+}-sepharose was prepared by 3 times washing with MiliQ water. The supernatant was added to the Ni^{2+}-sepharose and mixed for 1

hour at 4°C, then the suspension was transferred to the column and eluent flew through. The column was washed with the first washing buffer (50 mM NaCl, 10% Glycerol, 0.02% DDM, 50 mM ammonium acetate, 30 mM imidazole at pH7.5). Then continuously washing with the second buffer (50 mM NaCl, 10% Glycerol, 0.02% DDM, 50 mM ammonium acetate, 50 mM histidine). Histidine was used instead of imidazole because of its similar structure and higher Lyp1 stability in the presence of histidine. The elution of protein was completed with a histidine concentration of 235 mM. Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 5 mM directly after elution. All chromatography purification steps were carried out at 4°C or on ice. Samples were taken after each washing steps and elution fractions for sodium dodecyl sulphate-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) measurements. The concentration of elution fractions were determined by NanoDrop ND-1000 spectrophotometer at an absorption of 280 nm. The corresponded molecular weight of Lyp1 protein is 93.7 kDa and the extinction coefficient (ε) is 126.110 $M^{-1}cm^{-1}$. The in-gel fluorescence was imaged using Fujifilm LAS-300. Afterwards, the gel was stained by Coomassie Brilliant Blue R-250 Dye and imaged again.

Liposome preparation

Lipid concentration of stock solutions were at 20 to 25 mg/mL. The mix ratio is shown in **Table 1** in the Appendix. The chloroform used to dissolve 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and ergosterol was removed by rotary evaporation at 40°C, 400 mbar. To dry and prevent oxidation, the lipids were treated under nitrogen flow for approximately 10 minutes. Lipids mixture was dissolved in diethyl ether followed by evaporation to vacuum and resuspended in lipid buffer A (50 mM ammonium-acetate at pH 7.5, 50 mM NaCl, room temperature) to a final concentration of 10 mg/mL. The homogenization of liposomes were completed by sonication with a Sonics Vibra Cell Sonicator at 4°C for 2 minutes with 15 seconds on and 45 seconds off (5-s break between each pules) while the amplitude was set as 70%. Then the lipid was aliquoted to 1 mL Eppendorf tubes followed by three rounds of freeze in liquid nitrogen and thaw in warm water. The lipid aliquots were stored in liquid nitrogen afterwards.

Reconstitution to proteoliposomes

With the combination of the highest size-exclusion chromatography (SEC) concentration fractions, each 10 mg sample of lipids, 25 µg of purified Lyp1 were used (lipid-to-protein ration

is 400). 1 mL lipid stock in lipid buffer A was extruded through a 400 nm polycarbonate filter for 11 times. Then lipids were diluted again with lipid buffer A to 2 mL and titrated 65% of the OD_{540} of the initial value with Triton X-100. After the addition of purified Lyp1, 150 mg of wet BioBeads SM-200 were sequentially added after 15, 30 and 60 minutes and overnight nudation at 4°C. Bio-beads was filtered through a 10 mL plastic column and washed with 1 mL lipid buffer A. Afterwards, the mixture was ultracentrifuged for 20 minutes at 4°C, 80,000 rpm. The pellets were resuspended in 2.5 mL desired buffer and aliquoted in 1 mL. The proteoliposomes were stored in liquid nitrogen after three cycles of freezing and thawing.

Membrane scaffold protein (MSP) production and purification

Experiments were performed with *Escherichia coli* BL21(DE3). 100 µg/mL DNase, 1 mM PMSF and 1% TritonX-100 were added to 100 mL of cells (corresponding to 1 L of culture). The cells were broken by Maximator and then centrifuged for 30 minutes at 4°C, 30,000 × *g*. 20 mM imidazole was then added to the separated supernatant. To get a column volume (CV) of 7.5 mL, 15 mL 50% Ni^{2+}-sepharose was added to a large Biorad glass column. Then the column was washed with 4 CV demiwater and 4 CV 50 mM potassium phosphate at pH 7.8. Gently, the column containing the supernatant and 20 mM imidazole was incubated for 1 hour at 4°C while agitating. Then the column was washed first by washing buffer 1 containing 40 mM Tris, pH 8.0, 0.3 M NaCl and 1% Triton X-100, followed by washing buffer 2 (40 mM Tris, pH 8.0, 0.3 M NaCl, 50 mM Na-cholate and 20 mM imidazole). Then MSP_{1E3D1} was eluted by 40 mM Tris, pH 8.0, 0.3 M NaCl and 500 mM imidazole. The protein concentration was determined by NanoDrop with the absorbance at 280 nm, while the corresponded molecular weight is 32.6 kDa and extinction coefficient (ε) is 29400 $M^{-1}cm^{-1}$. SDS-Page in gel fluorescence samples were taken after each wash and elution to verify the purification (Figure A1).

^{14}C-lysine uptake assay

Each time 5 µg (Lyp1-GFP) of proteoliposomes was used in this assay. 3.2 µL Natamycin and same amount of N-Ethylmaleimide were incubated with 11.2 µL of liposomes before centrifugation. After the thawing and ultracentrifugation 20 minutes at 50,000 rpm at 4°C, proteoliposomes was extruded through a 400 nm membrane 11 cycles and resuspensed with inside buffer A (100 mM, pH 6.0, potassium acetate, 100 mM, pH 6.0, acetic acid, 10 mM pH

6.0 potassiumphosphate). The final concentration of reconstituted Lyp1 was 5 µg / 42 µL. Assays were performed at 30°C water bath under continuous magnetic stirring and at 1 µM ^{14}C-lysine. Samples were taken at a given time intervals as well as the total count. 70 µL of mixture containing radio-active lysine was taken from the reaction system each time and diluted in 2 mL outside buffer (110 mM, pH 6.0, sodium phosphate), and rapidly filtered through a 0.45 µm pore size nitrocellulose filter to recover the proteoliposomes. Filters were dissolved in Ultima Gold LSC cocktail, and radioactivity measured in a Tri-Carb 2800TR liquid scintillation analyzer.

Formation of proton membrane potential (Δψ)

To create membrane potential, proteoliposomes were diluted 28.5 times in the reaction solution. The outside buffer of the liposome was sodium phosphate at pH 6.0 while the internal one is potassium phosphate / potassium acetate buffer at pH 6.0. According to the formula provide, $\Delta\psi = -Z\log\frac{[K^+]_{in}}{[K^+]_{out}}$, $Z = \frac{2.3RT}{F}$, where R is gas constant, F is Faraday constant [2]. The actual potassium ion in-over-out ratio during the assay was depended on the degree of proteoliposome dilution into buffers. 0.5 µM valinomycin was added to each reaction system for exporting potassium ion, which can generally induce a positively charged outer membrane.

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Appendix

Mole percentage	1	2	3
POPC	27.5	20	10
POPS	35	35	35
POPE	35	35	35
Ergosterol	2.5	10	20

Table 1: Liposome components in mole percentage.

Inhibitors	**Slope gradient [pmol/min]**	**End concentration [µM]**
N-Ethylmaleimide	3.0847	10,000
Natamycin	1.7803	300
Control	3.0714	-
Desipramine	1.9037	200

Amitriptyline	1.6171	200
Thialysine	0.9257	20
L-Canavanine	2.7436	20

Table 2: Reaction speed and corresponded concentration of inhibitors.

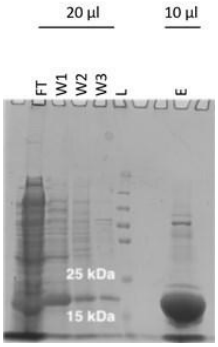

Figure A1: Purification SDS-PAGE gel results of MSP.