Role of the glutamic and aspartic residues in Na\(^+\)-ATPase function in the ZrENA1 gene of Zygosaccharomyces rouxii

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Abstract

The effect of replacement of negatively charged amino acid residues on the function of Na\(^+\) transport proteins of the salt-tolerant yeast Zygosaccharomyces rouxii was examined by heterologous expression of mutant proteins in a strain of Saccharomyces cerevisiae, RH16.6, that lacks native Na\(^+\)-ATPase activity due to null mutations of ENA1, ENA2, ENA3, and ENA4. Mutants of Na\(^+\)/H\(^+\) antiporter gene (ZrSOD2) and Na\(^+\)-ATPase gene (ZrENA1) of Z. rouxii were generated by site-directed mutagenesis. The significance of two aspartic residues arranged in tandem (D265 and D266) was demonstrated in Z. rouxii Na\(^+\)/H\(^+\) antiporter. Some Z. rouxii Na\(^+\)-ATPase mutant genes, namely E778A, D852A, and E981A present in the transmembrane domains (TMDs) and D736A, D743A, D748A, D749A, D759A, and D760A present in the cytoplasmic space were constructed. A lower level of salt tolerance was bestowed by the mutant genes D852A and E981A present in TMDs and D748A and D749A present in cytoplasmic space, compared with the wild-type gene (ZrENA1).

Keywords: Salt tolerance; Zygosaccharomyces rouxii; Na\(^+\)-ATPase; Negatively charged amino acid

1. Introduction

Traditional Japanese seasonings, such as miso paste and soy sauce, contain high salt concentrations, in particular NaCl. Yeasts playing a significant role in making these seasonings are most likely to be salt-tolerant, such as the salt-tolerant yeast Zygosaccharomyces rouxii. In order for yeast cells to grow in a high-salt-concentration environment, at least two mechanisms are required, one for adjusting the osmotic balance between cytoplasmic and extracellular fluids and another for extruding Na\(^+\) which may be toxic intracellularly [1].

In previous studies, we cloned and characterized two Na\(^+\)/H\(^+\) antiporter genes (ZrSOD2 and ZrSOD22) [2,3] and a Na\(^+\)-ATPase gene (ZrENA1) [4] from Z. rouxii, all of which encode factors involved in Na\(^+\) extrusion. Moreover, we have demonstrated that in Z. rouxii cells, Na\(^+\) extrusion is carried out via a Na\(^+\)/H\(^+\) antiporter, of which the driving force (H\(^+\) gradient) is established by the action of H\(^+\)-ATPase [2,3].

On the other hand, in Saccharomyces cerevisiae cells, Na\(^+\) extrusion is mainly carried out through the action of the Na\(^+\)-ATPase (gene ENA1) [5–7] with the Na\(^+\)/H\(^+\) antiporter (gene NHA1) playing only a negligible role [8–10].

On the basis of the findings reported to date, Na\(^+\)/H\(^+\) antiporter is the main driver of Na\(^+\) extrusion in the highly salt-tolerant yeast Z. rouxii, in the moderately salt-tolerant yeast S. cerevisiae it is the Na\(^+\)-ATPase, and in the salt-sensitive yeast Schizosaccharomyces pombe it is the Na\(^+\)/H\(^+\) antiporter. Therefore, no relationship between the salt tolerance of yeasts and the kind of Na\(^+\) extrusion machinery present is apparent. However, regardless of the different mechanisms involved, the extrusion of Na\(^+\) from cells is important for the salt tolerance of microorganisms.

S. cerevisiae RH16.6 strain has disrupted genes where four Na\(^+\)-ATPase genes (ENA1–ENA4) were deleted either totally or partially [6,7]. Thus, the ability of Na\(^+\) extrusion was lost in this strain. Since the cells of RH16.6 strain are sensitive to NaCl and LiCl, this strain is highly suitable for functional studies of membrane transport proteins that enable Na\(^+\) extrusion.

In a previous study, we examined the functionality of some Z. rouxii genes in S. cerevisiae RH16.6 cells.
the open reading frames (ORFs) of the \(Z.\) \textit{rouxii} \(\text{Na}^{+}/\text{H}^{+}\) antiporter genes \(\text{ZrSOD2}\) and \(\text{ZrSOD22}\) were inserted into an \(S.\) \textit{cerevisiae} expression vector, pYES2, and then expressed under the control of the \(\text{GAL1}\) promoter, a marked increase in the salt tolerance of transformant cells was observed [3]. Moreover, since the increased salt tolerance was dependent on the \(\text{pH}\) of the culture medium, the observed recovery of salt tolerance was deduced to be due to the action of the \(\text{Na}^{+}/\text{H}^{+}\) antiporter [3].

In addition, the ORF of the \(Z.\) \textit{rouxii} \(\text{Na}^{+}\)-ATPase gene, \(\text{ZrENA1}\), was also inserted into the pYES2 vector and the recombinant vector then introduced into RH16.6. The resulting transformants also displayed increased salt tolerance [4]. This increase in salt tolerance showed less dependence on medium \(\text{pH}\) than that of the \(\text{Na}^{+}/\text{H}^{+}\) antiporter gene, and was thought to occur via \(\text{Na}^{+}\) extrusion driven by \(\text{Na}^{+}\)-ATPase [4].

In the present study, we examined the effect of aspartic and glutamic residues present in the transmembrane domain (TMD) on the functions of the \(\text{Na}^{+}/\text{H}^{+}\) antiporter and \(\text{Na}^{+}\)-ATPase.

2. Materials and methods

2.1. Cultivation of yeast and bacteria

\(S.\) \textit{cerevisiae} RH16.6 (\(\text{MATa}\) \text{ura3-52} \text{leu2-3} \text{leu2-112} \text{his3} \text{Δ1} \text{tryp-280} \text{ena1::LUE2::ena4}\)) was cultured in uracil dropout medium [11]. \(E.\) \textit{coli} XL1-Blue

2.2. Recombinant plasmid and site-directed mutagenesis

The pYES2 vector harboring the ORF of the \(Z.\) \textit{rouxii} \(\text{Na}^{+}/\text{H}^{+}\) antiporter gene (\(\text{ZrSOD2}\)) or the \(Z.\) \textit{rouxii} \(\text{Na}^{+}\)-ATPase gene (\(\text{ZrENA1}\)) was constructed as previously reported [3,4]. Site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers (Stratagene) was cultured aerobically at 37°C in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) \(\text{NaCl}\), \(\text{pH}\) 7.2).

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used are shown in Fig. 1. The replacement of nucleotides was determined by DNA sequence analysis.

2.3. Transformation of S. cerevisiae RH16.6 and estimation of salt tolerance of transformants

The yeast transformation was performed using the alkaline cation yeast transformation kit (BIO 101) according to the manufacturer’s instructions. The estimation of salt tolerance of transformant cells was performed as described previously [3,4]. Briefly, the transformants were aerobically incubated at 30°C to stationary phase in uracil drop-out medium containing 2% raffinose. An aliquot (30 μl) of the culture was transferred to a medium (3 ml) containing 1% raffinose, 1% galactose, and various concentrations of NaCl or LiCl and lacking uracil; cell suspension was incubated aerobically at 30°C until the transformant growth in the absence of NaCl or LiCl was stationary. Cell growth was determined by measuring the optical density of each culture at 600 nm (OD_{600 nm}).

3. Results and discussion

3.1. Aspartic residues arranged in tandem were vital to Na^+/H^+ antiporter function

In *Vibrio alginolyticus* cells, aspartic residues arranged in tandem are important for the functionality of the Na^+/H^+ antiporter [12]. Examination of the amino acid sequence of the *Z. rouxii* Na^+/H^+ antiporter revealed aspartic residues arranged in tandem at amino acids (aa) 265 and 266. We then constructed separate mutant genes, in which either aspartate was changed to valine by site-directed mutagenesis. The salt tolerance of RH16.6 cells harboring these mutant genes (D265V and D266V) decreased to the level of control cells (RH16.6 cells harboring only the pYES2 vector, Fig. 2A). D265V and D266V cells showed similar tolerance to NaCl, but differed slightly in their tolerance to LiCl, D265V having a higher tolerance than D266V (Fig. 2B). These results suggest that D265 and D266 are equally important in the extrusion of Na^+ from cytoplasmic fluid to extracellular fluid, but that D265 is particularly important for Li^+ transport.

These two aspartic residues are present in TMDs of Na^+/H^+ antiporter, suggesting that the negatively charged amino acids in TMDs are vital to the functioning of Na^+-ATPase.

The RH16.6 strain becomes salt-sensitive due to a decrease in the ability of Na^+ extrusion caused by disruption of the Na^+-ATPase genes (*ENA1*-*ENA4*) [6,7]. In the present study, the expression of the Na^+/H^+ antiporter gene (*ZsOD2*) increased the salt tolerance to NaCl, sug-

3.2. Identification of negatively charged amino acid residues (aspartate and glutamate) as significant for the function of Z. rouxii Na^+-ATPase

In order for Na^+ ions to cross the cell membrane through Na^+-ATPase, a route (pore) formed by aggrega-
tion of its 10 TMDs is most likely required (see Fig. 4). The significance of negatively charged amino acid residues in moving Na\(^+\) through the pore is readily apparent. Moreover, we presumed that the residues present in the cytoplasmic space are related to the capture of Na\(^+\), and that the residues in extracellular space are for releasing Na\(^+\). Aspartic and glutamic residues present in a peptide not in the TMD but localized in cytoplasmic space may be important for Na\(^+\) capture.

We produced multiple alignments of five amino acid sequences of Na\(^+\)-ATPase cloned from yeasts *S. cerevisiae* (Ena1p and Ena2p) [5–7], *Z. rouxii* (Zrena1p) [4], and *Schwanniomyces occidentalis* (Soena1p and Soena2p) [13]. Fig. 3 shows the amino acid sequence of Zrena1p, with conserved residues in bold italics. Ten TMDs are also shown. Amino acid residues conserved in all five yeast Na\(^+\)-ATPases included glutamate at aa 778 (E778), aspartate at aa 852 (D852), and glutamate at aa 981 (E981) in the TMD, and aspartate at aa 736 (D736), aspartate at aa 743 (D743), aspartate at aa 748 (D748) and aa 749 (D749), and aspartates at aa 759 (D759) and aa 760 (D760) in the cytoplasmic space. The positions of the examined negatively charged amino acid residues are shown in Fig. 4.

Mutant genes containing a mutation at either one of these residues were constructed by site-directed mutagenesis (the residues arranged in tandem were converted simultaneously) and then introduced into RH16.6 strain using the pYES2 vector.

Na\(^+\) extrusion was inferred from the complementation level of the salt-sensitivity of RH16.6 cells by expression of the mutant gene. Fig. 5A shows the results obtained by analysis of the aspartic or glutamic residue present in the TMDs. The salt tolerance of RH16.6 cells harboring just one of two mutant genes (D852A and E981A) decreased to the levels of the pYES2 control cells. On the other hand, virtually no difference in salt tolerance was observed between E778A cells and cells harboring the wild-type *Z. rouxii* Na\(^+\)-ATPase (pZrENA1). E778 might not participate in the function of *Z. rouxii* Na\(^+\)-ATPase, probably because of the side chain of E778 not being oriented to the inner side of the Na\(^+\) pore.

Fig. 5B shows the results obtained by analysis of residues present in the cytoplasmic space. Among the four groups of negatively charged residues, it was only when aspartates at aa 748 and aa 749 were converted simultaneously to alanine that a significant decrease in salt tolerance was observed compared to pZrENA1 cells. These results suggest that besides two negatively charged residues (D852 and E981) present in the TMD, two aspartic residues (D748 and D749) present in tandem in the cytoplasmic space might also be related to Na\(^+\)-ATPase function (possibly Na\(^+\) extrusion).

The significance of the cytoplasmic aspartic residues D748 and D749 may lie in the capture of Na\(^+\) ions. In particular, D748 and D749 may be important for neutralizing positively charged amino acid residues present on the cytoplasmic side. Specifically, Zrena1p has a histidine res-
idue (H63) on the cytoplasmic side of the first TMD (TMD1) and two arginine residues (R921 and R922) on the cytoplasmic side of TMD8. Because these positively charged amino acid residues may affect the access of Na+ for agonistic effect, the neutralization of these three positively charged residues by the presence of negatively charged residues (e.g. D748 and D749) might be important for moving Na+ ions through the Na+ -ATPase molecule.

The negatively charged amino acid residues analyzed in the present study were only those conserved in some yeast Na+ -ATPase proteins. Therefore, the participation of other negatively charged residues in the functioning of Na+ -ATPase cannot be discarded.

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References