Protein Amino Acid Composition of Plasma Membranes Affects Membrane Fluidity and Thereby Ethanol Tolerance in a Self-flocculating Fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*

膜蛋白氨基酸组成通过改变膜流动性影响粟酒裂殖酵母和酿酒酵母融合株耐酒精能力

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摘 要 实验显示，一种氨基酸混合液（含异亮氨酸、甲硫氨酸和苯丙氨酸，添加浓度分别为 1.0、0.5 和 2.0g/L）能显著提高自絮凝酵母——粟酒裂殖酵母和酿酒酵母融合株 SPSC 的耐酒精能力。实验将菌体分别培养于添加（试验组）和未添加（对照组）该氨基酸混合液的条件下，然后收集菌体进行酒精（20%，*V*/*V*）冲击试验（30℃，9h），结果，试验组的菌体尚有一半以上的存活细胞，而对照组的菌体全部死亡。通过对试验组和对照组的菌体细胞膜蛋白质氨基酸组成分析发现，试验组的菌体耐酒精能力提高与所添加氨基酸组入菌体的细胞膜密切相关。以 DPH 为荧光探针的细胞膜流动性测定分析进一步揭示，氨基酸组入菌体的细胞膜后，细胞膜能有效抵抗高浓度酒精冲击诱发的膜流动性的提高，从而维持膜的稳定。因此，实验首次揭示膜蛋白氨基酸组成可通过改变膜流动性而影响酵母菌的耐酒精能力。

关键词 耐酒精，细胞膜流动性，膜蛋白氨基酸组成
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Abstract A combination of three amino acids including 1.0 g/L isoleucine, 0.5 g/L methionine and 2.0 g/L phenylalanine was found to enhance ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. When subjected to 20% (*V*/*V*) ethanol for 9 h at 30℃, all cells died whereas 57% remained viable for the cells grown in the presence of the three amino acids. Based on the analysis of protein amino acid composition of plasma membranes and the determination of plasma membrane fluidity by measuring fluorescence anisotropy using diphenylhexatriene as a probe, it was found that the significantly increased ethanol tolerance of cells grown with the three amino acids was due to the incorporation of the supplementary amino acids into the plasma membranes, thus resulting in enhanced ability of the plasma membranes to

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efficiently counteract the fluidizing effect of ethanol when subjected to ethanol stress. This is the first time to report that plasma membrane fluidity can be influenced by protein amino acid composition of plasma membranes.

Key words ethanol tolerance, plasma membrane fluidity, protein amino acid composition

There has always been considerable interest with respect to the production of fuel alcohol from renewable resource. However, to make ethanol an economically feasible biofuel, the process has to be optimized in terms of yield as well as ethanol production rate[1-3]. Fuel ethanol production would be improved by increasing ethanol tolerance of *Saccharomyces cerevisiae*. Significant improvements of alcoholic fermentation as a result of the addition of unsaturated fatty acids and sterols have been reported[4,5], their effect being attributed to the enhancement of resistance of yeast cells to ethanol.

There are many reports related to ethanol stress which suggest a relationship between plasma membrane lipid composition and ethanol tolerance[6,7]. Among various membrane components, unsaturated fatty acids and ergosterol are considered to be determinants of ethanol tolerance in yeast. However, little is known about the role of protein amino acid composition of plasma membranes in yeast ethanol tolerance. In this work, the effect of exogenously supplemented amino acids on the tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to ethanol was examined. Investigation was focused on understanding of the mechanism underlying the positive effect of added amino acids. The results presented in this paper demonstrate, for the first time, that protein amino acid composition of plasma membranes can significantly affect membrane fluidity and thus ethanol tolerance.

1 MATERIALS AND METHODS

1.1 Strain, media and culture conditions

The strain employed in this study was a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (termed fusant SPSC), which was constructed via protoplast fusion and preserved by our laboratory[8].

Agar slope medium for culture maintenance was composed of (g/L): glucose 10, yeast extract 3.85, peptone 3, agar 20. Growth medium for yeast aggregates formation contained (g/L): glucose 30, yeast extract 3.85, peptone 3. Fermentation medium for batch ethanol production consisted of (g/L): glucose 200, yeast extract 5, peptone 3. To form yeast aggregates, a loopful of cells from agar slope were inoculated into 100-mL volumes of growth media and cultivated on a rotary shaker at 30℃ for 18 h.

1.2 Determination of ethanol concentration

Ethanol concentration was determined by gas chromatography (Agilent 6890A GC, USA, equipped with flame ionization detector) as previously described[9]. The temperature of the inlet and of the detector was 160 ℃ and 230℃ respectively, and oven was operated isothermically at 90℃. The carrier gas was N_2 and n-butanol was used as the internal standard.

1.3 Measurement of viability

Cells grown in the presence or absence of a combination of three amino acids including 1.0 g/L isoleucine, 0.5 g/L methionine and 2.0 g/L phenylalanine, were harvested, washed twice with distilled water and then suspended in 20% (*V*/*V*) ethanol at 30℃. At suitable intervals, samples were taken in order to follow the concentrations of viable cells. Viable cells were counted by plating appropriate dilutions of cells on agar slope media and incubating at 30℃ for 2 or 3 days. Relative viability (%) = (C_t/C_0) × 100%, where C_0 and C_t represent number of viable cells per mL of cell suspension at the onset and a given time of incubation with ethanol respectively.

1.4 Isolation of plasma membranes

Late exponential phase cells were harvested, washed twice with distilled water and then twice with 1.2mol/L sorbitol solution. Cells were converted to spheroplasts essentially as described by Dickinson & Isenberg[10]. Spheroplasts were then harvested by low-speed centrifugation (700 ~ 800 g) and washed twice with 1.2mol/L sorbitol solution. The total membrane fraction was obtained as a pellet after osmotic lysis of the spheroplasts in 0.9% NaCl and centrifugation at 29000 g for 20min at 4℃. The plasma membranes were further purified according to the method of Schibeci *et al*[11].

1.5 Analysis of protein amino acid composition of plasma membranes

Plasma membranes were put into a chloroform/methanol solution (2:1, *V*/*V*) and stirred for 2.5h. The extracts were washed with a 0.88% (*W*/*V*) KCl solution and allowed to separate overnight. The top layers were analyzed with respect

to amino acids by the method of Albers *et al*[12].

1.6 Fluorescence measurements

Cells exposed to ethanol stress were washed and resuspended in phosphate buffer (pH 6.0). DPH (1,6-diphenyl-1,3,5-hexatriene) prepared in tetrahydrofuran was added to the cell suspension to a final concentration of 2μmol/L and then incubated in the dark at 30℃ for 45min. Excitation and emission wavelengths were 358 and 426 nm respectively. Steady-state fluorescence anisotropy (1/membrane fluidity) of DPH was calculated according to Sperka-Gottlieb *et al*[13].

1.7 Statistical methods

In this study, all experiments were carried out in triplicate and all determinations were done at least twice with mean values presented.

2 RESULTS AND DISCUSSION

Appropriate amino acids intended to use as a supplement for examining the impact on ethanol tolerance of cells were determined based on batch ethanol fermentation employing fusant SPSC: addition of three amino acids including 1.0g/L isoleucine, 0.5g/L methionine and 2.0g/L phenylalanine resulted in the highest final ethanol concentration in broth (data not shown).

Measurement of growth in the presence of ethanol is the most widely used method for determining ethanol tolerance[6]. Figure 1 illustrates the enhancing impact of the

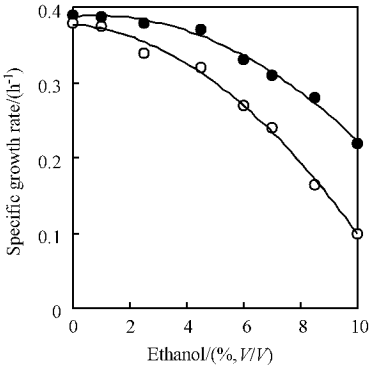

Fig. 1 Inhibition of specific growth rate of fusant SPSC at 30℃ by increasing concentrations of ethanol added to growth medium

Closed and open symbols represent growth medium supplemented with and without three amino acids including 1.0g/L isoleucine, 0.5 g/L methionine and 2.0 g /L phenylalanine respectively.

three amino acids on the resistance of fusant SPSC to ethanol-induced inhibition of the specific growth rate. Although sustaining maximal growth did not require the three amino acids, growth in the presence of ethanol was significantly improved by the addition of these three amino acids. The role of the three amino acids in ethanol tolerance of this strain was further examined with the result shown in Figure 2. After 9 h of exposure to ethanol, 57% and 0% of viability levels remained for the cells grown with and without the three amino acids respectively. This shows that the three amino acids are able to significantly enhance the tolerance of this strain to ethanol. What is the mechanism underlying the increasing effect of the three amino acids?

Enhancements in ethanol tolerance of *Saccharomyces cerevisiae* as a result of the incorporation of supplements such as unsaturated fatty acids and ergosterol into the plasma

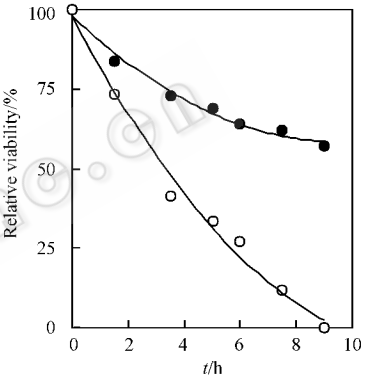

Fig. 2 Viability of fusant SPSC subjected to 20% (*V*/*V*) ethanol at 30℃

Closed and open symbols represent cells grown with and without three amino acids including 1.0g/L isoleucine, 0.5g/L methionine and 2.0g/L phenylalanine respectively before exposure to ethanol.

membranes of cells have been reported[4, 5]. So, we tried to check whether the three amino acids used as a supplement in this study to enhance ethanol tolerance of fusant SPSC were incorporated into the plasma membranes of cells. As shown in Table 1, cells grown with the three amino acids were exactly enriched in isoleucine, methionine and phenylalanine in their plasma membranes as compared with cells grown in the absence of this supplement, indicating that the added amino acids were incorporated into the plasma membranes of cells.

Shin *et al*[14] reported that supplementing *Saccharomyces sake* fermentation with albumin hydrolysate brought about a more than 60% increase in final ethanol concentration (144 g/L compared with 88 g/L without supplementation) due to an enhanced ethanol tolerance of cells grown with the supplement. However, the mechanism underlying the enhancing effect of albumin hydrolysate has not yet been explored. Therefore, we would like to know how the

incorporation of the three amino acids into the plasma membranes leads to the increased ethanol tolerance of cells. Since alterations in protein amino acid composition of plasma membranes may affect membrane properties, it was of interest to examine any change in membrane fluidity. As shown in Figure 3, in the absence of ethanol, fluorescence anisotropy (1/ membrane fluidity) of cells with plasma membranes enriched in the three amino acids increased in comparison with the control, suggesting that the incorporation of the three amino acids into the plasma membranes leads to the decreases in membrane fluidity. On the other hand, ethanol stress resulted in the increase in membrane fluidity. Increasing concentrations of ethanol led to increased membrane fluidity, regardless of what conditions under which cells were grown. However, the percentage of increase in membrane fluidity per unit of ethanol was lower for cells grown with the three amino acids as compared with the control. Consequently, in the presence of equal concentrations of ethanol, plasma membranes enriched with the three amino acids always maintained a lower membrane fluidity than the control; especially at high concentrations of ethanol, their differences became even more significant. These data suggest that the incorporation of the three amino acids into the plasma membranes results in the enhanced ability of fusant SPSC to counteract the fluidizing effect of ethanol, thus maintaining plasma membrane integrity.

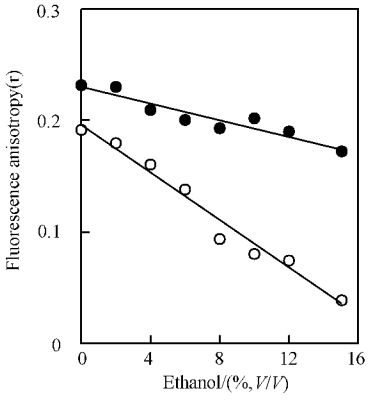

Fig. 3 Fluorescence anisotropy (r) values of DPH inserted into membranes of fusant SPSC cells following exposure to different concentrations of ethanol

Closed and open symbols represent cells grown with and without (control) three amino acids including 1.0g/L isoleucine, 0.5g/L methionine and 2.0 g/L phenylalanine respectively before exposure to ethanol.

It is generally accepted that the ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) in membrane lipids plays a key role in determining membrane fluidity. For example, in microorganisms, including yeasts, higher environmental temperatures generally produce an increase in the degree of saturation in membrane lipid (a reduction in UFA/SFA), which results in a decrease in membrane fluidity[15]. The results reported in this study demonstrate, for the first time, that protein amino acid composition of plasma membranes can significantly affect membrane fluidity: a marked increase in the contents of isoleucine, methionine and phenylalanine leads to a decrease in membrane fluidity. Plasma membranes enriched in the three amino acids acquire greater ability to counteract the fluidizing effect of ethanol when subjected to ethanol stress. This may be a novel strategy responsible for the increased ethanol tolerance of cells.

Table 1 Protein amino acid composition of plasma membranes of fusant SPSC cells grown under different conditions[a]

Amino acid	Composition/%	
	Ⅰ	Ⅱ
Alanine	7.65	10.81
Arginine	3.45	4.26
Aspartic acid	3.09	5.13
Cysteine	0.56	0.66
Glutamic acid	8.31	10.05
Glycine	9.00	9.03
Histidine	1.23	1.96
Isoleucine	10.17	4.96
Leucine	8.28	9.99
Lysine	6.60	8.25
Methionine	6.75	2.55
Phenylalanine	10.87	4.20
Proline	4.53	6.51
Serine	5.74	6.20
Threonine	4.02	4.09
Tyrosine	3.30	3.03
Valine	7.41	8.37

[a] Ⅰ and Ⅱ represent cells grown with and without three amino acids including 1.0g/L isoleucine, 0.5g/L methionine and 2.0g/L phenylalanine respectively.

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大力发展生物质能及其产业化

生物质能既有它的传统性，又在不断地发展和更新，广开其源。时值今日，它仍有大力发展之势，各个国家都很重视它。根据国内外对生物质能研发的现状和发展趋势，通过对它现实和长远发展战略的思考，有以下 10 个方面的启示。

(1) 化石燃料如石油为非再生能源，储量有限，未来将供不应求。据有关专家预测，到 2020 年我国缺 1.6 亿吨～2.2 亿吨，因此思考多途径开发新能源或替代能源很有必要。

(2) 使用化石燃料、煤炭有种种弊端，排放的有害气体污染环境日趋严重，如我国排放的 CO_2 居世界第二，而 SO_2 排放居世界第一。发展生物质能可大大减轻上述种种弊端。

(3) 生物质能是一大类可再生能源，洁净、来源广。包括农业秸秆在内所有有机废弃物都可得到充分利用；“取之不尽，用之不竭”，可从中获取生物能源，微生物技术的有效应用能发挥很大作用。

(4) 缓解当前我国能源短缺问题。我国进口石油的依存度 40%，开发生物质能及其它洁净能源非常有必要，同时也可确保能源的安全。

(5) 发展循环经济所必需。同时又促进生物质能的发展，有利于经济持续发展，有利于环境保护，有利于维持生态平衡。

(6) 发展生物质能拓宽农业新领域，即发展能源农业，将成为农业领域的新“战场”，有利于服务“三农”，更重要的是为农民增收开辟新途径。

(7) 发展粮食作物与发展能源作物具有很强的互补性，相互促进，推动“两作物”的发展，而生物质能也得到更稳健的发展；与此同时，不可忽视木本能源植物及其废弃物的有效利用，微生物技术有很大的应用潜力。

(8) 发展生物质能可减轻温室效应带来的危害，温室气体减排，有利于保护大气环境和促进生态平衡，造福于民。

(9) 发展生物质能实质上也是节约能源(石油)的一项重要举措。从长远计，应给予足够的重视，强化对这一领域的研究与开发。

(10) 使用生物质能效力所面临的问题或障碍需要有针对性地采取相应对策，使其产品有经济效益、市场竞争力，逐步走向国际化。现代生物技术－微生物技术的应用可加快生物质能发展的步伐，但对其基础及应用研究仍不可忽视。

总之，根据当前全球能源(石油)较为紧张的局面，油价呈上涨趋势。在我国，宜实施“两条腿走路”的方针，除发掘新油气资源和提高石油采收率之外，从长远计，需大力研究开发生物质能或可再生能源，走可持续发展的道路。

（柯　为）