Structural Changes and Inactivation of Saccharomyces cerevisiae in Grape Juice Induced by High Hydrostatic Pressure

고혈압에 의한 포도주의 Saccharomyces cerevisiae 구조적 변화와 사멸효과

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국문요약

포도는 생산기에서 널리 소비되는 과실로 포도 과피에 존재하는 천연색소인 flavonoids는 항산 플레스테롤 함량 저하, 항암적성, 항염성, 항바이러스성, 뇌졸중의 생리적 기능이 있다고 알려져 있다. 최근에 들어와 이들 과실주스 사용에 열려있는 레스버미르로 자란 포도주의 영양성분, 맛과 항기 개선을 위한 고혈압 치료에 관한 연구가 활발하게 이루어지고 있다. 본 연구는 포도주에서 문제가 되고 있는 ethanolic spoilage균인 S. cerevisiae의 고혈압 식균 효과와 세포 구조적 형태를 연구하였다. 1.2×10⁹ cfu/ml의 S. cerevisiae를 포도주에 접촉하고 24시간 비양하여 암반한 high barrier주머니에 20ml씩 넣고 20℃에서 200-600 MPa 조건으로 0-30분 동안 고혈압 장치로 실시하였다. 생산수는 YM agar로 poured 방법으로 실시하였으며 200 MPa에서 5, 10, 15, 20분 후의 생균수는 각각 2.2×10⁴, 4.5×10⁴, 2.8×10⁵, 9.8×10⁴, 9.5×10⁴ cfu/ml로 testing 현상을 관찰하였고, 400 MPa에서 5분 후 급격하게 감소하였다. S. cerevisiae의 사멸속도는 고혈압 치료가 높음수록 증가했으며 세포 손상도는 급속과 처리시간이 길수록 증가하였고. 이를 조건에 따른 효과 세포의 구조적 변화를 scanning electron microscopy와 transmission electron microscopy로 하였다. S. cerevisiae 세포는 압력에 의한 pinhole, surface roughening을 발견하였고, 세포 내부의 세포질, 이금, 핵 손상과 세포질 물질의 압력에 의하여 세포벽으로 이동하여 내부가 비어있는 현상을 관찰하였다.

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I. Introduction

New technologies such as non-thermal physical treatment processes are being developed to inactivate spoilage and pathogenic microorganisms. Traditional thermal processing, including pasteurization and retorting, can result in adverse changes to the flavor, taste, and nutrient content of foods. High pressure processing has been investigated as a method to stabilize citrus juices at reduced temperatures by inactivating the native microflora (1,3,7,13,16,18,22,23,27,31,34). The technology appears to successfully eliminate native microorganisms capable of causing spoilage in refrigerated citrus juices. Microbial decomposition of fruit juices is most frequently associated with fermentative yeasts such as *S. cerevisiae* which causes ethanolic spoilage, carbonation, production of hydrogen sulfide, and other off-odor (14,17). Since fruits are relatively low in protein and starch, there have been no sensory quality changes resulting from protein denaturation and the swelling of starch mixtures caused by exposure to high pressure. Successful inactivation of microorganisms by high hydrostatic pressure has been reported through numerous researchers (2-18). Many factors are reported to influence the microbial inactivation such as effective pressure strength and duration (1,8), temperature of treated food (28), and enzymatic inactivation of treated foods (5,12,16,21,26,29,32).

The physical phenomenon responsible for microbiological inactivation is less clear. The most popular theory describing pressure inactivation of microorganism was recently reviewed by Castro *et al.* (3), in which pore formation and destruction of the semipermeable membrane barrier are described. Changes to the *S. cerevisiae* cells observed by scanning electron microscopy (SEM) after pressure treatment, showing surface roughening and pinholes (25,30), along with craters or holes, elongation, roughening, wrinkling, and increased bud scar formation (24,25). Reports on transmission electron microscopy (TEM) techniques to observe high pressure-treated yeast cells are scarce. TEM methodologies were previously utilized to observe budding and cell wall rupture (31) of *S. cerevisiae* in studies not involving high pressure. Thus, the objective of this study was to utilize SEM and TEM to observe structural changes of *S. cerevisiae* with high hydrostatic pressure.

II. Materials and Methods

1. High pressure equipment

A Cold Isostatic Press manufactured by ABB Autoclave (Quintus Food Processor 6, Columbus, OH, USA), consisting of an 800 milliliters pressure vessel, a piston-type intensifier, and an external hydraulic pump, was used. A water jacket was installed around the pressure vessel, and the temperature of the pressure medium (water) was maintained at 20°C. A thermocouple was placed inside the vessel to measure the temperature of the pressure medium during high pressure treatment.

2. Test strains

The strains of *S. cerevisiae* (ATCC 166664, Rockville, MD, USA) yeast cells were cultured in yeast malt broth (DIFCO 0711-01-9, Detroit, MI, USA) at 25°C.

3. Preparation of medium

Reactivated *S. cerevisiae* cell was inoculated at 2×10^6 cfu/ml in grape juice (pH 3.4, 12% sucrose)
and incubated for 2 days at 25°C.

4. High pressure treatment

Polyethylene bags were washed using 2% (w/w) 
H$_2$O$_2$ solution and dried in a sterilized clean bench 
under UV light. Twenty milliliters of grape juice 
medium was transferred into the polyethylene bags 
(45 mm x 120 mm) and the bags were then heat-
sealed without entrapping air bubbles. The bags 
were put into the pressure vessel and pressurized at 
200-600 MPa for 1-20 min at 20°C. After high 
pressure treatment, the bags were removed and 
held in ice-water for further analysis.

5. Enumeration of survivors

The high pressure-treated samples were kept at 
4°C to minimize microbial growth. The survivor 
cell counts were determined by plating 1 ml of 
diluted samples onto duplicate plates of YM agar 
(Difco Laboratory, Detroit, MI, USA). Colonies were 
counted after 2 days of incubation at 25°C.

6. Electron microscope observation

SEM and TEM were performed on the microbial 
suspensions after high pressure treatment. For SEM 
observeration, samples were treated, fixed, and 
dehydrated following the same method as for both 
SEM and TEM observations. Critical point drying of 
the yeast cells were accomplished by washing three 
times with 100% hexamethyldisilazane (HMDS) for 
15 min. The gold-coated sample was observed with 
a JSM-5410-LV SEM (JEOL, Tokyo, Japan). For TEM, 
both untreated and treated cells of S. cerevisiae were 
centrifuged at 4000 x g for 10 min at 10°C, and the 
supernatant was discarded. The cells were 
resuspended in 2 ml of 2% paraformaldehyde and 
2% glutaraldehyde in 0.05 M sodium cacodylate 
buffer (pH 7.4) and fixed for 24 h at 4°C. The 
microcentrifuge tube contents were centrifuged at 
a constant 10,000 x g for 20 s in Beckman Model 
Microfuge E (Palo Alto, CA, USA).

The supernatant was discarded, and the pellet 
was resuspended in 0.05 M sodium cacodylate 
buffer (pH 7.4). The 1 mm$^3$ yeast cell cubes were 
infiltred in 10 ml Spurr resin, and the resin was 
replaced every 2 h for 24 h on a 5 rpm rotator titled 
at a 45 angle. Polymerization of Spurrs resin to form 
specimen blocks was induced heating the resin in 
molds at 70°C for 8 h. The specimen blocks formed 
were hand trimmed with a razor blade and 
sectioned into a thickness of approximately 0.5μm 
with a Reichert Model OM2(Austria) ultra 
microtome. The sections were stained with 2% 
uranyl acetate and Reynolds lead citrate, and 
viewed with JEM 1010 TEM (JEOL, Tokyo, Japan) 
transmission electron microscope operated at 80 kV.

III. Results and Discussion

1. High pressure inactivation of Saccharomyces 
cerevisiae

The S. cerevisiae population was reduced from an 
initial concentration of 2.2 x 10$^7$ cfu/ml to total 
inactivation. Figure 1 shows the effect of high 
pressure on inactivation of S. cerevisiae. Under high 
pressure at 200 MPa, the number of survivors was 
reduced to 4.5 x 10$^4$ cfu/ml for 5 min. However S. 
cerevisiae suspended in grape juice was completely 
inactivated at 400 MPa. In the application of high 
pressure processing, which is a batch type process, 
the duration of treatment exerts a critical influence 
on the cost. The duration of high pressure treatment 
in the following experiments was limited to less
than 20 min (Fig. 2). The survivor cell number of *S. cerevisiae* suspended in grape juice was reduced to $10^3$ cfu/ml for 5 min and "tailing" phenomenon was observed at 200 MPa. However, a further reduction completely inactivated the cells at 400 MPa and 20°C. These results were similar to the inactivation in *S. cerevisiae* reported by Ogawa *et al.* (21). They showed that the reduction of the total microflora in Satsuma mandarin orange juice by 5 log CFU/ml required pressure treatments of 350 MPa for 30 min or 400 MPa for 5 min.

2. Changes in the ultrastructure of *S. cerevisiae* by high pressure treatment

Untreated (control) *S. cerevisiae* cells inoculated in sterile grape juice for 20 min and viewed with TEM exhibited cellular organelles including the nuclei, vacuoles, and cytoplasmic materials (Fig. 3-A). After pressure treatment of *S. cerevisiae* inoculated in grape juice, the micrograph of yeast cell structure exhibited cytoplasmic shrinkage, cytoplasmic compartmentalization, and cellular organelle disruption (Figs. 4-B, C, and D).

Harrison *et al.* (14) showed after high voltage pulsed electric fields (PEF) treatment of *S. cerevisiae* inoculated in apple juice, the cytoplasmic material parted from the cell wall and cell wall voids. Shin *et al.* (30) also observed that PEF treatment in Lactobacillus plantarum shrinks the cytoplasmic membrane away from the outer membrane, and the blank space separated inner materials from the membrane.

In our observations, it was evident that the structural changes in yeast cells of cytoplasmic material shrinkage toward the cell wall and voided cytoplasmic are caused by the pressure. Osumi (24) reported on the inner structure of *S. cerevisiae* treated with hydrostatic pressures of 100, 200, 300, 400, and 500 MPa, and the apparition of nucleus was 14, 60, 81, 90, and 100%, respectively.

SEM micrographs of both the control and the pressurized cells are shown in Fig. 4. *S. cerevisiae* cells treated at pressures lower than 600 MPa at 20°C, showed slight effects on the outer shape, pinholes (Fig. 4-A), surface roughening (Fig. 4-C), and cell wall rupture (Fig. 4-D). Osumi *et al.* (25) reported that *S. cerevisiae* cells treated to pressure lower than 400 MPa at room temperature showed a slight effect on the outer shape, but the bud scar area of the cell wall disrupted and/or damaged was at pressure higher than 500 MPa. It is difficult to
Fig. 3. Transmission electron microscope of high pressure treated S. cerevisiae in grape juice for 5 min at 20°C. (A) untreated control cell, showing nucleus(N), nucleus membrane(NM), vacuole(V), cell membrane(CM) and cell wall(CW); (B) pressure-treated cell at 200MPa; (C) 400MPa; (D) 600MPa. Bar corresponds to 500nm (______).
identify the effects of high pressure inactivation because complex damage could be observed after the high pressure treatment. Sohn (31) predicted high pressure inactivation has two effects, one is the destruction of the cell structure by physical effect of high pressure such as adiabatic expansion during high pressure treatment and the other by protein denaturation under high pressure. From these observation it is suggested that the structural effects of hydrostatic pressure on *S. cerevisiae* occurred on the membrane system particularly on the cell membrane invagination in the cytoplasm.

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