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Inhibition of Microbial Growth and Enrichment of γ-Aminobutyric Acid during Germination of Brown Rice by Electrolyzed Oxidizing Water

ZHAN-HUI LU,[1] YAN ZHANG,[1] LI-TE LI,[1] REMPEL B. CURTIS,[2] XIAO-LIN KONG,[3] R. GARY FULCHER,[4] GONG ZHANG,[5]* AND WEI CAO[6]*

[1]*Department of Food Sciences & Engineering and* [6]*Department of Bio-environmental Engineering, China Agricultural University, Beijing 100083, China;* [2]*Richardson Center for Functional Foods and Nutraceuticals, Manitoba, Canada R3T 6C5;* [3]*Anhui Agricultural University, Hefei, Anhui 230036, China;* [4]*Department of Food Science, University of Manitoba, Manitoba, Canada R3T 2N2; and* [5]*University of Winnipeg, Manitoba, Canada R3T 6A5*

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ABSTRACT

Electrolyzed oxidizing water (EOW) has been regarded as a potential environmentally friendly broad spectrum microbial decontaminant. EOW with a pH of 3.0 and oxidation reduction potential of 1,079.0 mV were generated by the electrolysis of a dilute NaCl solution (20 mM) in an electrochemical cell. The effects of EOW, 1% NaClO solution, and alkaline electrolyzed water on controlling microbial growth, germination ratio, and enrichment of γ-aminobutyric acid in germinated brown rice (GBR) were evaluated in this study. Results show that EOW was the most effective at inhibiting microbial growth during germination. Rinsing the rice grains with EOW at 12-h intervals resulted in aerobic plate count reductions of 4.82 log CFU/g, while soaking resulted in bacterial count reductions of 5.38 log CFU/g after 72 h of germination. Moreover, EOW significantly enriched γ-aminobutyric acid content in GBR ($P < 0.05$); content was increased 1.6 times in grain rinsed with EOW and 1.8 times in grain soaked in EOW. The findings indicate that EOW is a feasible disinfectant for industrial GBR production.

Germinated brown rice (GBR), which originated in Japan, has a high nutritional value. Brown rice grains are soaked in water to promote germination, and during this process γ-aminobutyric acid (GABA) accumulates *(9)*. GABA is a free amino acid produced primarily by the decarboxylation of L-glutamic acid (Glu), catalyzed by glutamate decarboxylase (GAD, EC 4.1.1.15) *(14)*. GABA plays an important role as a neurotransmitter in the brain and spinal cord of mammals *(13)*; other functions of GABA include induction of hypotensive effects, diuretic effects, and tranquilizing effects *(6, 9)*. Cooked rice is a staple food in Asian countries and is consumed daily. Easy to cook and with a texture softer than that of brown rice, GBR has had great success in Japan and other Asian countries, and its consumption has increased rapidly in recent years.

The sprouting process usually occurs in a humid, warm environment, which also provides suitable conditions for the growth of pathogens. Microbiological surveys have shown the presence of a variety of foodborne pathogens in sprouts. *Escherichia coli* O157:H7, various serotypes of *Salmonella,* and *Bacillus cereus* have been the causative agents of documented outbreaks of foodborne illness associated with sprouts *(5)*. In all of the reported outbreaks, the likely source of contamination was seed. Brown rice was given a surface-disinfection treatment to avoid the growth of microorganisms during the processing of GBR: soaking in 1% sodium hypochlorite solution for 10 min resulted in a 2.0-log decrease by aerobic plate count in culture water after 1 h of germination; soaking in 10% sodium hypochlorite solution for 10 min was found to inhibit germination *(18)*.

Electrolyzed oxidizing water (EOW), also known as acidic electrolyzed water, is an effective and environmentally friendly disinfectant for sprouts *(2, 15)*. Compared with chemical antibacterial treatments, EOW, produced from pure water and NaCl (99% pure table salt), has a less adverse environmental impact *(7, 17)*. EOW is usually generated by electrolysis of a dilute NaCl solution in a cell with anode and cathode electrodes separated by a membrane; EOW is obtained from the anode side. EOW with a low pH value (<3.0), a high oxidation reduction potential (ORP; >1,000 mV), and containing free chlorine has been proven to exhibit strong bactericidal activity against foodborne pathogens *(7)*. Alkaline electrolyzed water (AlEW, also known as electrolyzed reducing water), generated at the cathode side, has a detergent effect on organic compounds *(4)*. Several studies have shown that EOW can be effective in reducing pathogenic bacteria on the surface of fruits and vegetables *(10–12)* and in food processing facilities *(1, 3)*. EOW with a pH of 2.6, an ORP of 1,150 mV, and about 50 mg/liter free chlorine resulted in *E. coli* O157:H7 reductions of 0.22 to 1.56 log CFU/g on alfalfa seeds and 1.05 to 2.72 log CFU/g on sprouts, and did not cause any visible damage to the sprouts *(17)*. In 2002, EOW was approved as an indirect food additive in Japan *(4)*. However, there has been no specific research on the

* Author for correspondence. Tel: (86)10-62737569; Fax: (86)10-62737570; E-mail: caowei@cau.edu.cn; GZHANG@manitoba-ehealth.ca.

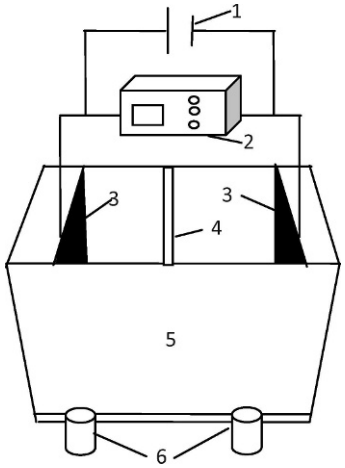


FIGURE 1. *Schematic diagram of the experimental electrolyzed water generator. (1) DC power; (2) controller; (3) electrodes; (4) membrane; (5) electrolytic cell; (6) outlets.*

effects of EOW on controlling microbial growth during germination of brown rice.

The objectives of this study were to evaluate the efficiency of electrolyzed oxidizing water for reducing microbial contamination during brown rice germination and to determine the effects of EOW on GABA contents in GBR. The potential of germination enhancement and GABA enrichment by alkaline electrolyzed water was also investigated.

MATERIALS AND METHODS

Materials. The paddy rice used was a nonwaxy *Japonica* rice (cultivar Yu Ying 6) harvested from the farm of Henan Academy of Agricultural Sciences, Henan, China, in August 2008. The paddy rice was dried in a convection oven at 40°C to a final moisture content of 13.6% dry basis. Dried paddy rice (300 g) was dehusked in an experimental rubber roll sheller (THU class 35A, Satake rice machine, Tokyo, Japan). From the resultant brown rice, 210-g samples were polished using a laboratory model abrasive roller polisher (TM-05, Satake grain testing mill). A good polish of 8% was given to the samples to obtain milled rice. The milled rice samples were manually separated to exclude broken and cracked grains. The brown rice and milled rice samples were sealed in plastic bags and stored at 4°C before use. The standards of GABA and Glu were purchased from Sigma (St. Louis, MO). Analytical grade chemicals and distilled water were used in this study.

Preparation of treatment solutions. EOW and AlEW were generated by electrolysis of 20 mM NaCl solution at 20 V and 12 A for 15 min using an experimental electrolyzed water generator (model ZSJ-1, Shenyang Dongyu Xinbor Technology Company Ltd., Shenyang, China) that consisted of an electrolysis cell in which anode and cathode electrodes were separated by a membrane (Fig. 1). EOW with a pH value of 3.0, an ORP of 1,079.0 mV, and an available chlorine concentration of 25 mg/liter was collected from the anode side of the generator, and AlEW (pH 11.0, ORP −763.0 mV) was obtained from the cathode side. The physicochemical properties of electrolyzed water were measured immediately after preparation. The pH and ORP values were measured using a dual scale pH/ORP meter (HM-30R, DKK-TOA Corporation, Tokyo, Japan) with a pH electrode (GST-5741C, DKK-TOA Corporation) and an ORP electrode (PST-5721C, DKK-TOA Corporation). The available chlorine concentration was determined by a colorimetric method using a digital chlorine test kit (RC-2Z, Kasahara Chemical Instruments Corp., Saitama, Japan). The detection limit is 0 to 300 mg/liter. All measurements were made in triplicate.

In the meantime, a NaClO solution containing 38 mg/liter of available chlorine was diluted in sterile distilled water to obtain a fresh 0.1% (vol/vol) NaClO solution. Sterile distilled water was used as a control.

Evaluation of microbial growth and Glu and GABA content during germination. Twenty grams of brown rice was washed with 300 ml of distilled water three times, and then the grains were spread on a sterile perforated tray lined with four layers of cheesecloth and incubated in darkness at 37°C for 0, 12, 24, and 36 h, respectively; the grains were rinsed every 12 h with 300 ml of sterile distilled water. At each time interval, the rice grains were collected aseptically and immediately used for microbial analyses. The grains incubated for 0 h were obtained by placing the rice grain on the cheesecloth and collecting immediately. The collected rice grains (5 g) were weighed into 100 ml of sterile physiological saline solution. The flask was shaken vigorously by hand to rinse the microbial cells from the surface of the rice grains, and then 1 ml of the solution was aspirated aseptically and mixed with a 10-fold amount of sterile physiological saline solution (containing 0.85% NaCl and 0.1% Bacto Peptone). For aerobic plate count, 1 ml of the mixture was serially diluted (1:10) in the sterile saline solution, and appropriately diluted solutions (0.1 ml) were surface plated in triplicate on plate count agar (Merck, Darmstadt, Germany) and incubated at 37°C for 48 h before counting. In addition, the GBR sample incubated for 36 h was dried in a laminar flow hood at room temperature (21 ± 1°C) for 24 h, and then the content of Glu and GABA was determined by a method described below.

For inhibition of microbial growth by different treatments during germination, the following two procedures were used.

Rinsing procedure. The washed brown rice was soaked in 500 ml of treatment solutions in darkness at 37°C for 12 h before being spread on the sterile perforated tray. During 72 h of incubation, the rice was rinsed by the corresponding freshly prepared solution at 12-h intervals. After germination, approximately 5 g of germinated rice grains were aseptically collected for microbial analysis. The others were used to count visually the percentage of germinated rice grains. The length of root and sprout of germinated grains were directly measured. Then the grains were dried in the same way as above and analyzed to determine the content of Glu and GABA.

Soaking procedure. The procedure was almost the same as rinsing, except the grains were placed on four layers of cheesecloth and immersed in 500 ml of different treatment solutions at 37°C for 84 h. The treatment solutions were changed to the freshly prepared ones at 12-h intervals. All treatments were replicated three times.

HPLC analysis of Glu and GABA. Glu and GABA were extracted and analyzed by high-performance liquid chromatography (HPLC) *(16)*. The dried rice grains were ground into powder with a Wiley mill (200 mesh, model 1093 Cyclotec sample mill, Foss Tecator, Eden Prairie, MN) and then added to four volumes of cold 70% ethanol. After storage overnight at 4°C, the samples were centrifuged (34,800 × *g*, 20 min), and the pellets were washed twice with 70% ethanol. The supernatants were pooled and

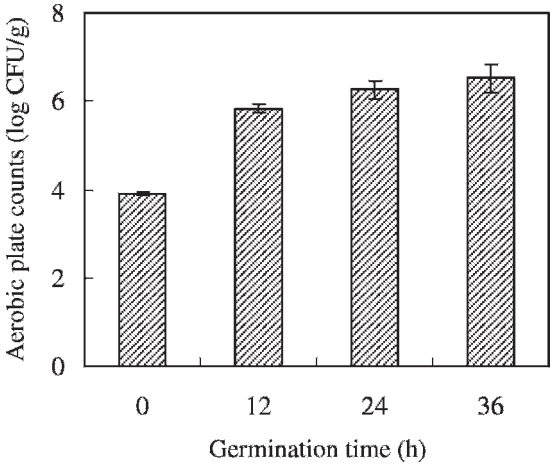


FIGURE 2. *Aerobic plate counts on the surface of brown rice grains during germination.*

concentrated under vacuum and finally stored in a deep freezer at −20°C. The Glu and GABA contents of the extracts were analyzed by a HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization. Buffer A (0.1 M ammonium acetate, pH 6.5) and buffer B (0.1 M ammonium acetate containing acetonitrile and methanol, 44:46:10 [by volume], pH 6.5) were used. For sample preparation, a 50-μl aliquot of extract was removed and dried under vacuum (37°C, 20 mm Hg). Then 20 μl of a first coupling reagent (methanol-water-triethylamine [TEA; 2:2:1, by volume]) was added. After mixing, the sample was directly dried under vacuum for 10 min and then reacted with 30 μl of PITC reagent (methanol-PITC-TEA-water, 7:1:1:1 [by volume]) at room temperature for 20 min before drying under vacuum to remove PITC. The derivatized samples were then redissolved in 500 μl of buffer A (used as mobile phase for HPLC) and filtered through a Millipore membrane (0.22 μm). A 20-μl sample was injected into the HPLC system (Shimadzu, Kyoto, Japan), consisting of an LC-10AT pump, a UV detector (SPD-10AVVP), and a C_{18} reversed-phase column (Dikma Diamonsil C_{18} column ϕ 4.6 by 250 mm, Dima Co., Ltd., Orlando, Finland), using a gradient system of buffer A (100 to 0% after 50 min) and buffer B (0 to 100% after 50 min). The operating temperature was 43°C, and the absorption was measured at 254 nm. Quantitative data for GABA and Glu were obtained by comparison to known standards *(16)*.

Statistical analysis. All samples were measured in triplicate. One-way analysis of variance was conducted with an SAS package (version 6.12 for Windows, SAS Institute Inc., Cary, NC). Tukey's multiple range test was used to determine the significant differences among the means at the 5% probability level.

RESULTS

Microbial growth during brown rice germination. Figure 2 shows the microbial growth during a normal germination condition of brown rice (37°C for 36 h). The aerobic plate counts were 3.91 log CFU/g for brown rice at the beginning of germination. It increased rapidly during the first 12 h and achieved 6.52 log CFU/g after 36 h germination. The GABA content of brown rice was 6.79 mg/100 g (dry basis) and increased significantly to 14.44 mg/100 g after 36 h of germination ($P < 0.05$) (Table 1). In contrast, the Glu content of GBR was less than that of the brown rice, indicating that Glu was consumed during germination. There was much less GABA and Glu in milled rice.

TABLE 2. *Aerobic plate counts showing the effect of different treatments during germination on inhibition of microbial growth on brown rice grains*[a]

Treatment	Rinsed (72 h, rinsing at a 12-h interval)	Soaked (84 h, replacing fresh solution at a 12-h interval)
Electrolyzed oxidizing water	1.43 ± 0.05 B	0.48 ± 0.06 B
1% NaClO	2.00 ± 0.13 B	0.78 ± 0.28 B
Alkaline electrolyzed water	6.07 ± 0.33 A	5.15 ± 0.17 A
Distilled water (control)	6.25 ± 0.23 A	5.86 ± 0.34 A

[a] Values are means ± standard deviations (log CFU per gram), $n = 3$. Means in the same columns followed by different letters are significantly different as determined by Tukey's multiple range test ($P < 0.05$).

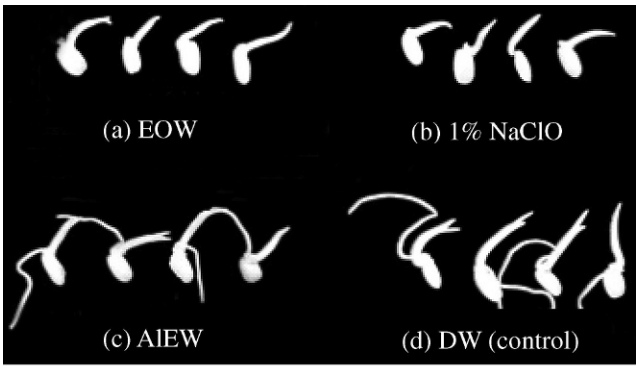
Effects of different solutions on microbial inhibition and GABA accumulation during germination. Table 2 shows the inhibition of microbial growth effected by rinsing or soaking brown rice during germination with EOW, AlEW, and 1% NaClO treatments. Soaking was more effective than rinsing for all tested solutions. EOW and 1% NaClO were both effective inhibitors of microbial growth during brown rice germination; 72 h of rinsing or soaking by EOW resulted in an aerobic plate count reduction of 4.82 and 5.38 log CFU/g, respectively. However, AlEW had no bactericidal effect on microbial growth during brown rice germination, and in the control samples, there was almost no reduction in aerobic plate count.

Table 3 shows Glu and GABA contents in germinated brown rice after different treatments. Treatment with AlEW increased the Glu content of the rice grains significantly ($P < 0.05$), although GABA content showed no significant difference compared with the control (Table 3). However, EOW not only inhibited microbial growth but also significantly enriched GABA in GBR ($P < 0.05$).

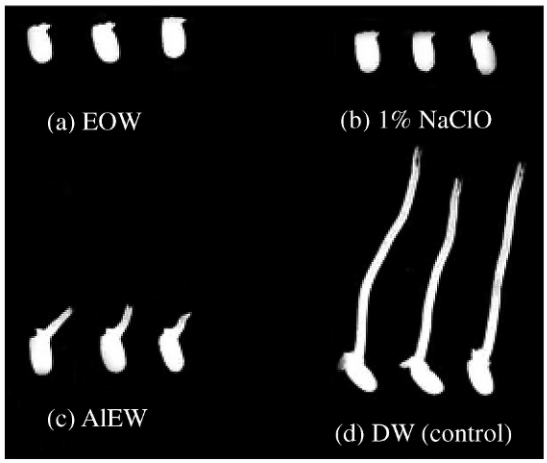
TABLE 1. *Glu and GABA contents of brown, milled, and germinated brown rice*[a]

Content	Brown rice	Milled rice	Germinated brown rice
γ-Aminobutyric acid (GABA)	6.79 ± 0.92 B	1.83 ± 0.26 C	14.44 ± 0.15 A
Glutamic acid (Glu)	16.72 ± 0.32 A	3.70 ± 1.08 C	11.28 ± 0.35 B

[a] Values are means ± standard deviations (milligrams per 100 g, dry basis), $n = 3$. Means in the same row followed by different letters are significantly different as determined by Tukey's multiple range test ($P < 0.05$).



(A) Rinsed



(B) Soaked

FIGURE 3. *Germinated brown rice incubated in different solutions. (A) Rinsed; (B) Soaked. EW, electrolyzed water; DW, distilled water.*

Compared with the control, GABA content increased 1.6 times in brown rice rinsed with EOW and 1.8 times in rice soaked in EOW. Although, similar to treatment with EOW, treatment with 1% NaClO solution inhibited microbial growth, it had an adverse effect on accumulation of GABA.

Effects of treatment on germination ratio and length of sprout and root of GBR. Figure 3 shows photos of GBR treated by rinsing or soaking with EOW, 1% NaClO, AlEW, and distilled water. The resulting root length, sprout length, and germination ratio of GBR are shown in Table 4. EOW and 1% NaClO treatments inhibited growth of root and sprout, and they decreased germination ratio by about 3 and 7%, respectively, especially by soaking ($P < 0.05$). Root growth was inhibited completely when rice was soaked in EOW.

DISCUSSION

Figure 2 illustrates the serious health problem of microbial growth during brown rice germination. While germination increased the GABA content of GBR by 2.1 and 7.9 times compared with brown rice and milled rice, respectively, the naturally occurring microorganisms also propagated rapidly, up to 6.52 log CFU/g during the germination process (Table 1 and Fig. 2). EOW treatment showed the strongest inhibition of microbial growth during germination (Table 2), reducing aerobic plate counts by 4.82 to 5.38 log CFU/g when it was used to rinse or soak the rice grains. In addition, EOW had a pronounced positive effect on GABA accumulation, which is very important for manufacturing functional foods rich in GABA (Table 3). A significant decrease of Glu content was noted with EOW treatment, suggesting that EOW enhanced the transmission from Glu to GABA. The mechanism is unclear but will be an interesting topic for further studies. These findings, which to our knowledge have not previously been reported, support the use of EOW for surface disinfection of brown rice as a safer alternative to the use of aqueous sodium hypochlorite.

In industrial production, the main purpose of brown rice germination is to enrich GABA, but the sprouts can easily detach from the rice kernels during packaging, resulting in a poor appearance and texture of the cooked rice. Table 4 shows the strong inhibition on growth of roots and sprouts of brown rice treated by EOW. EOW treatment produces GBR with a minimum bud, no root, no bacteria contamination, and increased GABA, thus enhancing its commercial value. A germination rate of 85% is regarded as acceptable and is chosen as the criterion for the selection of treatment conditions in germination tests *(8)*. In this study, the germination ratio of brown rice treated with EOW was higher than 91.5% and thus can be accepted.

The observation that soaking seeds in AlEW prior to germination could increase germination ratio had lacked supporting evidence. This study showed that the germination ratio of brown rice was significantly increased by rinsing or soaking with AlEW, with rinsing giving better results than soaking (Table 4 and Fig. 3). However, because

TABLE 3. *Glu and GABA content in germinated brown rice after different treatments*[a]

Treatment	Glu		GABA	
	Rinsed	Soaked	Rinsed	Soaked
Electrolyzed oxidizing water	7.78 ± 1.21 D	7.61 ± 1.85 C	19.54 ± 1.53 A	16.25 ± 1.29 A
1% NaClO	11.38 ± 1.06 B	8.40 ± 0.58 C	11.33 ± 0.01 C	7.91 ± 1.94 C
Alkaline electrolyzed water	13.85 ± 1.02 A	14.80 ± 1.22 A	13.03 ± 0.44 B	10.52 ± 1.63 B
Distilled water (control)	9.84 ± 0.08 C	11.00 ± 0.98 B	12.17 ± 0.46 B	9.09 ± 0.17 B

[a] Values are means ± standard deviations (milligrams per 100 g), $n = 3$. Means in the same column followed by different letters are significantly different as determined by Tukey's multiple range test ($P < 0.05$).

TABLE 4. *Effect of different treatments on the germination of brown rice grains*[a]

Treatment	Length of sprout (mm)		Length of root (mm)		Percentage of germination	
	Rinsed	Soaked	Rinsed	Soaked	Rinsed	Soaked
Electrolyzed oxidizing water	7.86 ± 1.26 B	1.08 ± 0.29 C	1.29 ± 0.68 C	ND	92.7 ± 1.7 C	91.5 ± 0.7 C
1% NaClO	8.42 ± 1.24 B	0.81 ± 0.14 C	1.86 ± 3.94 C	ND	89.2 ± 2.3 C	86.3 ± 1.9 D
Alkaline electrolyzed water	12.03 ± 1.68 A	4.15 ± 0.90 B	22.18 ± 0.81 B	0.2 ± 0.06	99.5 ± 1.3 A	97.1 ± 0.8 A
Distilled water (control)	12.08 ± 1.34 A	27.98 ± 5.11 A	28.18 ± 4.94 A	0.3 ± 0.07	95.8 ± 0.9 B	94.4 ± 1.1 B

[a] Values are means ± standard deviations, $n = 3$. Means in the same column followed by different letters are significantly different as determined by Tukey's multiple range test ($P < 0.05$). ND, under detection level.

AlEW treatment did not increase GABA content or inhibit microbial growth during germination, its application in GBR production is limited (Table 3).

In conclusion, this study demonstrated that EOW is an effective disinfectant for inhibition of microbial growth during brown rice germination, and it strongly enriches GABA content in the final product. Because it is also safer for the environment, EOW is a feasible antimicrobial agent to replace aqueous sodium hypochlorite in the production of GBR.

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