The arginine deiminase pathway of koji bacteria is involved in ethyl carbamate precursor production in soy sauce

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Abstract
Ethyl carbamate (EC) is a group 2A carcinogen generated from a few precursors in many fermented foods and alcoholic beverages. Citrulline, urea, carbamoyl phosphate, and ethanol are common precursors detected in fermented foods. In this study, citrulline was proved to be the main EC precursor in soy sauce, which was found to be accumulated in moromi mash period and correlated with the utilization of arginine by koji bacteria. Six koji isolates belonging to three genera were identified to be able to accumulate citrulline via the arginine deiminase (ADI) pathway. Among these strains, only Pediococcus acidilactici retained high activities in synthesis and accumulation of citrulline in the presence of high concentration of sodium chloride. These results suggested that P. acidilactici is responsible for the accumulation of citrulline, one of the EC precursors, in the process of soy sauce fermentation.

Introduction
Ethyl carbamate (EC) is a byproduct generated during fermentation process that belongs to the 2A group of carcinogen (Beland et al., 2005; Coulon et al., 2006). It is widely persisted in brandy, bread, yogurt, Chinese rice wine, and traditional condiment soy sauce (Battaglia et al., 1990; Matsudo et al., 1993; European Food Safety Authority, 2007; Koh & Kwon, 2007; Weber & Sharypov, 2009). EC is formed from various precursors (Ough et al., 1988) through a reaction promoted by heat and acid condition with ethanol (Kitamoto et al., 1991). Urea and citrulline have been confirmed as the main precursors of EC in alcoholic beverages and other fermented foods (Mira de Orduña et al., 2000). Most EC precursors were accumulated through the growth of microorganisms during fermentation process. For the purpose of eliminating the risk of foods and beverages, it is of great importance to confirm the main precursors of EC in fermented products and the corresponding microbial metabolic pathways.

The precursors of EC and the formation pathway of these precursors are not exactly the same for fermented foods and beverages. The presence of EC in sake was confirmed to be contributed by sake yeast through urea synthesis (Kitamoto et al., 1991), while citrulline was proposed as the main precursor of EC in wine and soy sauce (Matsudo et al., 1993; Mira de Orduña et al., 2000). The major route of citrulline accumulation in anaerobes from foods and beverages was the arginine deiminase (ADI) pathway (Liu et al., 1996). ADI pathway is composed of three critical enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC), and carbamate kinase (CK), that are encoded by the arc operon (arcA, arcB, arcC etc.). These enzymes catalyzed three reactions as shown below (Zuniga et al., 2002). Accumulation of citrulline in fermented foods is mainly the consequence of microbial decomposing of arginine and synthesis of ornithine that catalyzed by ADI (arcA) and OTC (arcB), respectively (Liu et al., 1996; Mira de Orduña et al., 2000).
Environmental factors such as pH, NaCl, temperature, and ethanol affect citrulline accumulation through regulation of gene expression (Vrancken et al., 2009a, b; Araque et al., 2013). Expression of arc operon is tightly regulated in anaerobic bacteria. The key genes of arc operon were shown to have different expression levels in Pseudomonas (Gamper et al., 1991) with normal culture condition. The expression level of arcA and arcB could dramatically influence the excretion of citrulline and ornithine. In addition to ADI and OTC, activity of a putative transporter which is responsible for re-uptaking citrulline and excreting ornithine is also involved in citrulline accumulation (Liu et al., 1996; Liu & Pilone, 1998; Rimaux et al., 2013). The transport activity is affected by Na⁺ concentration (Indiveri et al., 1997). Therefore, investigation and comparison of environmental conditions effect on ADI pathway regulation is of great importance for revealing the factors causing citrulline accumulation and thereafter explain EC formation mechanism in fermented products.

Although pediococci were thought to have the ability to decompose arginine into citrulline through the ADI pathway in soy sauce (Matsudo et al., 1993), a convincing study should be performed to prove its relevance. Objectives of this study were to elucidate the responsible strains for EC production and the mechanism of EC production during soy sauce fermentation process.

### Materials and methods

#### Media

Lactic acid bacteria were grown on de Man Rogosa Sharpe (MRS) agar described by Balcázar et al. (2008), faculty anaerobes were cultivated on nutrient agar described by Rohban et al. (2009). Screening media was based on the media described by Chang & Chang (2012) (glucose (0.1%, w/v), tryptone (0.5%, w/v), yeast extract (0.5%, w/v), meat extract (0.5%, w/v), sodium chloride (0.25%, w/v), tween 80 (0.1%, w/v), MgSO₄ (0.02%, w/v), MnSO₄ (0.005%, w/v), FeSO₄ (0.004%, w/v), ammonium citrate (0.2%, w/v), K₂HPO₄ (0.2%, w/v), CaCO₃ (0.01%, w/v), bromocresol purple (0.006%, w/v), arginine (1.0%, w/v), and agar (2%, w/v), pH 5.3). Modified MRS broth was based on MRS broth with addition of 18% NaCl (w/v), 10 g L⁻¹ arginine.

#### Isolation of microorganisms from koji

Fresh koji samples collected from soy sauce plant in Southern China were mixed with 0.85% NaCl (w/v) at a ratio of 1 : 1.6 (w/v). Solutions were then serially diluted and plated onto MRS agar and nutrient agar. All cultures were cultivated at 37 °C (interior temperature of koji) for 24 h until single colonies appeared.

#### Screening of citrulline production microorganisms

Single colonies isolated from koji were subcultured on screening media for testing the ability of utilization of arginine. All plates were then incubated at 37 °C for 48 h. Strains that are able to synthesis citrulline from degrading arginine were indicated by formation of purple halo surrounding bacterial colonies. Citrulline-producing candidates were inoculated into screening media mentioned above without adding bromocresol and agar and static culture at 37 °C for 48 h. Citrulline accumulation assay was performed by diacetyl monoxime method. (Ballini et al., 2010).

#### Growth conditions and fermentation experiment

After koji isolates were grown in 50 mL MRS broth at 37 °C and static culture to the late-log/early stationary phase, as assessed by cell sedimentation, cells were harvested by centrifugation at 8000 g for 10 min at 4 °C. Cells were washed twice with pH 7.0 PBS washing buffer. Cell pellets were then resuspended in appropriate amounts of PBS buffer and pipetted into 50-mL modified MRS broth for static culture at 37 °C and pH 5.5 for 7 days.

#### Reaction of EC precursors with ethanol

Reactions were taken in a 0.2 M sodium acetate buffer, and the pH was adjusted to 4.8. Absolute ethanol was used to adjust the alcohol concentration. Heat treatment was conducted in temperature-regulated water baths. Citrulline and urea were heated under the sterilization condition of soy sauce (95 °C, 30 min) with different concentrations of ethanol.

#### Quantification of EC

The quantification method was similar to that of Wu et al. (2012). The RTX-WAX capillary column (30 m × 0.25 mm × 0.25 μm) and QP-2010 ultra gas chromatography/mass spectrometry (GC/MS) (Shimadzu, Suzhou,
China) were used for separation. EC standard was purchased from Sigma-Aldrich, and the purity was > 99%.

**Determination of urea and ethanol**

Urea and ethanol were determined as the methods of Matsudo et al. (1993).

**Determination of amino acids**

L-citrulline, L-ornithine monohydrochloride, and L-arginine were used as standards (Sangon Biotech, Shanghai, China). One milliliter of cell culture or raw soy sauce samples were centrifuged (10 000 g, 10 min) to remove cells, the supernatants were then diluted with trichloroacetic acid (5%, m/v) and filtered through a water phase membrane with 0.22 μm pore size (Sangon). Arginine, citrulline, and ornithine were quantified using a method described by Spano et al. (2009) by HPLC (Agilent HPLC-1200, Agilent Technologies, Wilmington, DE) equipped with an automatic sampler system. A 4.6 × 250 mm hypersil ODS-2 (Thermo Scientific, MA) was used for separation. The concentration of each amino acid was calculated by the area for a given amino acid of known concentration.

**DNA manipulations**

Genomic DNAs of the isolated strains were extracted with E.Z.N.A. Genomic DNA Isolation Kits (Omega Bio-Tek) according to the instructions of manufacturer.

PCRs were performed in a thermal cycler (Cycler C1000, Bio-Rad) with primers listed in Table 1. PCR products were purified and sent to Sangon for sequencing. 16S rDNA sequences were analyzed using BLAST searches (www.ncbi.nlm.nih.gov/BLAST/).

**Results**

**Determination of EC precursors in soy sauce**

To determine the main precursors of EC, concentration of urea, citrulline, and ethanol in raw soy sauce samples from different batches were firstly measured. Among three batches of raw soy sauce, urea, citrulline, and ethanol were determined to be 55 mg L\(^{-1}\), 2200 mg L\(^{-1}\), and 2% (w/v), respectively. Then, reactions comprised different concentrations of ethanol and precursors were set as described in Materials and Methods. As shown in Table 2, 50 mg L\(^{-1}\) urea and 2% ethanol generated 6.9 ppb EC, while combination of the same concentration of ethanol with 2000 mg L\(^{-1}\) citrulline yielded 32.1 ppb EC. This demonstrated that citrulline led to higher EC level when interacted with ethanol. EC concentration was also increased with the increasing of ethanol concentration. Furthermore, amount of EC generated with both 50 mg L\(^{-1}\) urea and 2000 mg L\(^{-1}\) citrulline almost equals to the EC level in raw soy sauce samples (37 ppb, data not shown) after heat treatment under the same condition. It is plausible that citrulline is the main precursor of EC in soy sauce, and the trend of citrulline accumulation during soy sauce fermentation is a clue to find out the crucial microorganisms and its corresponding pathway for citrulline accumulation.

**Formation of citrulline (EC precursor) during soy sauce fermentation**

Citrulline and twenty common amino acids in the moromi were detected from the very beginning of soy sauce fermentation to investigate the formation mechanism of citrulline. Except for arginine, yields of other common amino acids were all increased during the first week (data not shown). Citrulline rose from 0.1 g L\(^{-1}\) to 2.2 g L\(^{-1}\) in the early of moromi mash period before adding the yeast, and then, it remained at around 2.0 g L\(^{-1}\) until the end of fermentation (Fig. 1). Meanwhile, at the early stage of moromi mash period, arginine concentration decreased from 1.5 g L\(^{-1}\) to 0.9 g L\(^{-1}\). Given the fact that the increasing of citrulline along with reduction of arginine, accumulation of citrulline is probably the result of decomposition of arginine via the ADI pathway existed in anaerobic bacteria (Liu & Pilone, 1998).

**Characterization of citrulline metabolism of koji bacteria**

Bacteria from koji were observed to grow slowly in the condition with high concentration of sodium chloride.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'→3’)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-1492R</td>
<td>AAGAGTTTGATCCTGCTGCTMCAG</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>Ped-arcAF</td>
<td>ACTATATGGAGGCGCTGTTGA</td>
<td>P. acidilactici arcA</td>
</tr>
<tr>
<td>Ped-arcAR</td>
<td>ATCAAGGATTGCTGGATGTT</td>
<td>P. acidilactici arcB</td>
</tr>
<tr>
<td>Ped-arcBF</td>
<td>GCAGGCGCTGAAGGTAAG</td>
<td>P. acidilactici arcB</td>
</tr>
<tr>
<td>Ped-arcBR</td>
<td>GCTTCTGGAGATCTACTGAG</td>
<td>P. acidilactici arcC</td>
</tr>
<tr>
<td>Ped-arcCF</td>
<td>GGCCTGGGAATGCGCGATA</td>
<td>P. acidilactici arcC</td>
</tr>
<tr>
<td>Ped-arcCR</td>
<td>TGATTAACCGTCCGAGTT</td>
<td>P. acidilactici arcC</td>
</tr>
</tbody>
</table>

**Table 2. Formation of EC (ppb) with urea and citrulline**

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>Urea (50 mg L(^{-1}))</th>
<th>Citrulline (2000 mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>1.2</td>
<td>3.8</td>
</tr>
<tr>
<td>2%</td>
<td>7.0</td>
<td>32.1</td>
</tr>
<tr>
<td>4%</td>
<td>17.5</td>
<td>57.7</td>
</tr>
</tbody>
</table>
(15–20%, data not shown). It suggested that strains with the ability of converting arginine to citrulline in koji were potentially those citrulline producers in the moromi mash period. Therefore, koji was used as the source to isolate strains that can accumulate citrulline.

A total of 80 strains with different morphology panels were isolated from koji, among which 35 of them were cultivated on MRS agar; the rest 45 strains were obtained from nutrient agar. Among these isolates, only six strains from MRS agar and three strains from nutrient agar were detected to possess the ability to decompose arginine into citrulline (Table 3). These strains were identified to be Weissella confusa, Weissella cibaria, P. acidilactici (MRS medium), and Staphylococcus (nutrient agar). Strains from these species were commonly regarded as salt-tolerant strains in the manufacture of soy sauce (Tanaka et al., 2012), which gave the confidence in their ability of production of citrulline under saline condition. Interestingly, citrulline accumulation assay on these strains showed that P. acidilactici produced most citrulline (Table 3). This indicated that P. acidilactici was the crucial strain and played an important role in accumulation of citrulline during soy sauce production.

**Effect of NaCl on citrulline metabolism of koji microorganisms**

Addition of high-level saline (15–20%, w/v) was a necessary and important process in moromi mash period. It is reported that Weissella, Pediococcus, and Staphylococcus from koji and moromi all belonged to salt-stress tolerance genera. Therefore, the effect of presence of NaCl on citrulline accumulation was tested for isolates belong to weissella, pediococci, and staphylocci (Table 3). To further determine the citrulline contributor, the degree of citrulline accumulation was calculated for the strains isolated from koji when they were placed into osmotic stress environment. Table 3 showed that all strains exhibited a citrulline production in the presence of NaCl except for W. cibaria. However, in contrast to the control group without adding NaCl, only P. acidilactici produced significant amount of citrulline in 18% NaCl, the conversion rate from arginine to citrulline reached nearly 100%. This result further confirmed that P. acidilactici was the most responsible species for accumulation of citrulline in soy sauce.

**Discussion**

In this study, the major precursor of EC in soy sauce was confirmed to be citrulline. Subsequently, citrulline was proved to be correlated with the decomposition of arginine via the ADI pathway of anaerobes. Pediococcus acidilactici was then isolated from koji and was confirmed to be the crucial strain for citrulline accumulation in soy sauce for the first time. Furthermore, we confirmed the presence of high level of NaCl was a critical factor for citrulline production in koji.
environmental factor for its accumulation in the process of soy sauce fermentation.

*Pediococcus* was a common salt-tolerant species in soy sauce. It was considered to possess the ability of arginine degradation. Strains from this genus (*i.e.* *Pediococcus halophilus*, now resigned as *Tetragenococcus halophilus*) was assumed to have the ability of decomposing arginine to citrulline might be responsible for citrulline in soy sauce (Matsudo *et al.*, 1993). However, *T. halophilus* was found to be appeared when moromi mash period proceeded 2 weeks (Tanaka *et al.*, 2012). It means that *T. halophilus* neither became the predominant bacterium crowd during the first week of moromi mash period, nor produced large amounts of citrulline. Moreover, our study showed that *T. halophilus* did not accumulate citrulline through ADI pathway in the presence of high NaCl concentration (data not shown). Hence, *P. acidilactici* was confirmed to be responsible for the citrulline accumulation in soy sauce.

NaCl has been proved to inhibit the conversion of ornithine from citrulline (Vrancken *et al.*, 2009a). In this study, citrulline produced by *P. acidilactici* was found to be increased significantly in the presence of 18% (w/v) salt, while ornithine decreased remarkably. Conversion ratio of arginine to ornithine also decreased (Table 3) in the same condition. These results suggested that the conversion from citrulline to ornithine by *P. acidilactici* was inhibited by saline. Based on our results and previous studies, two preliminary conclusions could be assumed. One is that different expression level of *arcA* and *arcB* in ADI pathway may induce the changes of enzyme activity ratio of ADI and OTC and further caused the accumulation of citrulline. Araque *et al.* (2013) once proved that environmental factors such as pH and ethanol had effects on *arc* genes expression in *Lactobacillus brevis* and *Pediococcus pentosaceus*. *Pediococcus acidilactici* in this study contains three ADI pathway key genes (Fig. 2), the inhibition of conversion of citrulline to ornithine in *P. acidilactici* may be regulated by the *arc* operon in the presence of 18% NaCl. We hope to analyze the regulation of *arc* operon in response to NaCl stress via qRT-PCR to reveal ADI pathway regulation mechanism in the future. The other conclusion is that NaCl may inhibit citrulline reuptake from the outer membrane. When arginine was depleted, the extracellular citrulline would be further converted into ornithine, this process was considered to be controlled by a putative citrulline/ornithine antiporter (*C/O* antiporter). Citrulline was found to be no longer converted into ornithine after arginine depleted when the...
gene encoding C/O antiporter was knocked out in L. sakei CTC494 (Rimaux et al., 2013). Citrulline/ornithine exchange activity governed by C/O antiporter in rat liver mitochondria was also inhibited by Na⁺ (Indiveri et al., 1997). These evidences further demonstrated that the reuptake of citrulline was inhibited by NaCl, resulting in citrulline accumulation. The possible mechanism of ADI pathway regulation by NaCl was shown in Fig. 3. Influences of NaCl on citrulline accumulation indicated that high saline (industrial relevant) is a critical factor for citrulline accumulation. Therefore, a better control of the production process for keeping a low NaCl concentration such as the employment of low-salt solid state fermentation may be a good strategy to reduce the risk of foods.

This study provides a better understanding on citrulline accumulation mechanism and also contributes to improve the quality safety of soy sauce.

**Acknowledgements**

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