Effect of eliminating hdcA gene of Staphylococcus epidermidis TYH1 on Histamine production

Safoora Pashangeh¹, Enayat Berizi², Majid Majlesi³, Sajad Ghaderi³, Victor Nizet⁴, Samira Dahesh⁴

¹Department of Food Science and Technology, School of Agriculture, Jahrom University, Jahrom, Iran
²Department of Food Hygiene and Quality Control, School of Nutrition and Food Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
³Department of Nutrition, School of Health & Nutrition Sciences, Yasuj University of Medical Sciences, Yasuj, Iran
⁴Department of Pediatrics, School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, USA

ABSTRACT

Background and Objectives: The possible adverse effect of histamine on human health has made it a detrimental aspect to the quality and safety of many fermented food products especially fish sauce.

Materials and Methods: In the present study, hdcA gene in Staphylococcus epidermidis TYH1 was knocked out and its effect on histamine production was evaluated. HdcA encodes histidine decarboxylase, an enzyme that produces histamine from histidine. Both strains of TYH1, the wild type (WT) and mutant (∆hdcA) were then incubated in tryptic soy broth (TSB) supplemented with histidine (0.5 mM). The histamine content determined by capillary zone electrophoretic (CZE) analysis. Safety assessment of this mutant of food origin was conferred by virulence genes.

Results: It was found that S. epidermidis TYH1 exhibited production of histamine (50.09 ± 0.06 μg/mL), while ∆hdcA strain of TYH1 exhibited no histamine forming activity. Safety assessment of ∆hdcA revealed the presence of nuc gene, while superantigenic toxins and cot genes were not observed. Therefore, it has the ability to be used as a starter culture to decrease the histamine content in any fermented food products.

Conclusion: Our study findings may contribute to provide a novel approach of promoting the food safety of fish sauce and other fermented food products regarding the regulation of histamine content.

Keywords: Staphylococcus epidermidis; Histamine; Histidine decarboxylase; Capillary electrophoresis; Enterotoxin

INTRODUCTION

Histamine is a heterocyclic biogenic amine (BA) found in a variety of organisms and is formed by the bacterial decarboxylation of free histidine (histidine decarboxylase, hdc). It is known as a foodborne chemical hazard and causes scombroid poisoning (1).

Adverse effects of histamine on the health of consumers are including tachycardia, headache, flush, itch and decreasing in blood pressure (2). The toxic and defect action levels of histamine in fish products are 50 and 500 ppm respectively which established by FDA (3).

Foods containing high levels of histamine are fish,
seafood and fermented products (4). A popular and commonly used fermented fish product in South- east Asian countries is fish sauce, now consuming worldwide. It contains 20 g/L nitrogen, composed of 80% amino acids that introduced it as a source of dietary protein. The highest ever reported histamine concentration in fish sauce was 1220 ppm (5). High levels of free amino acids in fish and its fermented products resulted in bacterial decarboxylase activity during the insufficient refrigeration process. Therefore, possible methods for controlling histamine content of these products must be applied (6). Potential approaches of reducing histamine accumulation in foods have been proposed, including the prevention of histamine-producing bacterial activity, limiting the BAs constitutive amino acids by preventing the proteolytic activity, inhibition of amino acid decarboxylase activities and controlling the manufacturing process. The oxidative deamination of histamine by histamine oxidase has the potential to degrade it. This method could be applied by the addition of microorganisms or enzymes that decompose histamine (6-8). Since controlling the bacterial decarboxylase activity is more promising than the inhibition of histamine-producing bacteria and the oxidative activities of microbial enzymes and chemical compounds are insufficient for decreasing histamine to acceptable levels, we explored eliminating the histidine decarboxylase gene that governs the formation of histamine from histidine in preliminary trials. In this preliminary study, a mutant strain of S. epidermidis TYH1, a halotolerant histamine-producing bacterium isolated from Japanese fermented fish paste (fish-miso) (9), lacking hdcA gene was generated. Since staphylococci were frequently isolated from fermented fish products, it can be introduced as a fermenting organism able to decrease histamine content. Therefore safety assessment of this mutant of food origin was conferred by investigating the existence of virulence genes for possibility of using this mutant as a starter culture in fermented food products.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A new histamine forming strain of S. epidermidis TYH1 was originally isolated from fish-miso, kindly provided to our laboratory (9). Bacterial strains were cultured in TSB at 37°C in shaking incubator for 24 h. Chloramphenicol (20 μg/mL) was used for plasmid expression of hdcA or empty vector control. All the experiments were conducted with S. epidermidis TYH1, WT and AhdcA mutant strains containing the empty vector control as a proper comparison for experiments using the complemented strain. The diluted bacterial cultures (1:30 in fresh TSB) were grown to logarithmic phase $A_{600} = 0.4 (2 \times 10^8$ CFU/mL). The experiment conditions resulted in similar growth kinetic for all strains (9, 10).

Targeted mutagenesis and complementation vector construction. Exact allelic replacement of S. epidermidis TYH1 was conducted using PCR-based methods as described (10). Primer sequences were designed using the hdc gene of the S. epidermidis TYH1 characterized by Yokoi et al. (9). Briefly, 300 bp of 1/3 middle sequence of hdcA was PCR-amplified from chromosomal DNA of S. epidermidis TYH1 with the primers hdc-F+attB1, 5’-gggggacaaatttacaaaaagcaggctctgaagtctgatgtaec-3’ and hdc-R+attB1, 5’-gggggacacattttgacaagagctgggtcctgcatctcaacgaa-3’. The resulting PCR products were fused in a second round of PCR using oligonucleotide primers, hdc-F+attB1 and hdc-R+attB1, and were then cloned into the temperature-sensitive vector pKOR1 using BP clonase enzyme mix reaction (Invitrogen). The resulting plasmid pKOR1 was first electroporated into S. aureus RN4220 and then into S. epidermidis TYH1. PCR and sequence analysis are acknowledged the exact in-frame allelic replacement of the hdc gene which was settled by a two-step procedure of temperature change and antisense counterselection (10).

Histamine production by bacteria. S. epidermidis TYH1 was cultured overnight in 10 mL of TSB medium at 30°C. Aliquots (5 mL) were then transferred into 100 mL of fresh TSB and incubated for an additional 24 h at 30°C. Centrifuged bacterial pellet (10000 x g for 10 min) was washed with phosphate buffer (0.1 M, pH 6.0), adjusted to the final concentration of $2 \times 10^8$ cells/mL and then inoculated into histidine-supplemented medium (TSB containing 0.5 mM histidine (TSBH)). Recent culture was incubated for 24 h at 30°C. Following, after sedimentation (10000 x g for 5 min at 4°C) the collected supernatant was filtered through a 0.45 μm sterile filter membrane (8, 10).

Histamine analysis. Capillary zone electrophoretic analysis (CZE) was performed for determination
of histamine content using the method of Vitali et al. (11) with some modification. One mL of the prepared supernatant was mixed with 2 mL of 0.1 M HCl in a centrifuge tube. The mixture was homogenized using LabINCO vortex for 2 min (LabINCO, England). Subsequently, the supernatant was obtained by centrifugation at 10000g for 5 min (Eppendorf, Hamburg) at 25°C and filtered using a Whatman No. 1 filter paper. This procedure was duplicated for each residue. Both of the supernatants were mixed into a 5 mL microtube and filled up to 4 mL with 0.1 M HCl, filtered with a 0.45 µm filter and injected into the CZE-DAX system. Analysis was carried out by a CZE apparatus (Prince Autosample, Model I-Lift, 450 Series, Netherland) equipped with a UV-visible detector set at 210 nm, a temperature control device (set at 25°C) and data treatment software (Data Acquisition and Analysis Software, DAX). Calculation of peak area was used for the quantification of histamine in samples.

Safety assessment of knockout strain: DNA extraction. S. epidermidis TYH1 WT and ΔhdcA were inoculated in 2 mL of TSB at 30°C for 24 h. After centrifugation (5000 x g for 10 min) the harvested cells were washed with distilled water, suspended in 0.5 mL of TE-buffer containing 10 mM Tris-HCl; 1 mM EDTA (pH 8.0), and then 20% sodium dodecyl sulfate was used for cell lysis. The solution was boiled for 20 minutes, and then the cellular debris was removed by centrifugation (10000 x g for 5 minutes). The total DNA which obtained by centrifugation was precipitated with ethanol (70%) and used as template DNA for PCR (10).

Pathogenicity factors. S. epidermidis TYH1 WT and ΔhdcA mutant strains were examined for the presence of genes coding pathogenicity factors, including classical enterotoxin genes sea, seb, sec, sed, see, enterotoxin-like toxin Q gene (selq), and TSST-1 gene (tstl) (12), coagulase (13), and nuclease (14). Table 1 shows the PCR primer pairs. PCR amplifications were performed as described by Rahmdel et al. (15). For selq, tst1, coa and nuc detection, the uniplex PCR assay using each primer pair was applied. The reaction mixture (25 µL) contained 12.5 µL of Taq DNA Polymerase 2.0x Master Mix RED (1.5mM MgCl2; Ampliqon, Copenhagen, Denmark), 0.4 µM of each primer, and 50-100 ng DNA template. Superantigenic toxin genes, (SEs), were co-amplified by multiplex PCR according to Omoe et al. (16). The positive control strains used in this study were S. aureus DSM 19040 (sec, see), and S. aureus DSM 19041 (sea, seb, and sed).

RESULTS

Construction of hdcA deletion mutant. The homologous recombination method was applied for the construction of ΔhdcA mutant. As shown in Fig. 1, the targeted 300 bp bands, designed to validate the elimination of hdcA gene, were obtained from the genome of TYH1 WT, but they could not be obtained from the HdcA deletion mutant, ΔhdcA. Consequently the recombinant cassette fragment was successfully introduced into the locus of prosequence of TYH1 and the prosequence was replaced and the mutant strain TYH1 ΔhdcA was acquired and recognized by PCR.

Histamine production by bacteria. The histamine forming activity of TYH1 strains, WT and ΔhdcA, was tested by inoculating of resting cells in TSB containing 0.5 mM histidine. The histamine content was determined by the Capillary zone electrophoresis (CZE) method. Fig. 2 showed the histamine contents in WT and ΔhdcA strains of TYH1. S. epidermidis TYH1 exhibited production of histamine, forming histamine to about 50.09 ± 0.06 µg/mL, possessed histidine decarboxylase activity. ΔhdcA strain of TYH1 exhibited no histamine forming activity which is in agreement with the lack of hdcA gene.

Pathogenicity factors. To the virulence of HdcA deletion mutant strain of TYH1, ΔhdcA, a series of PCR assays were conducted. PCR analysis of genes coding for pathogenicity factors in the genome of S. epidermidis TYH1 have been summarized in Fig. 3. We investigated the staphylococcal pathogenicity factors in 2 categories: exotoxins and exoenzymes. Exotoxin genes including staphylococcal enterotoxins (SEs) and the SE-related toxin, toxic shock syndrome toxin-1 (TSST-1), are members of the superantigenic toxin. ΔhdcA did not harbor any of the superantigenic toxin genes. Exoenzymes, such as nuclease and coagulase were explored by the uniplex PCR assay using each primer pair. Amplification with nuc primers revealed that ΔhdcA was positive for amplification of a 270-300 bp specific band, corresponding to nuc gene. However, more virulent exoenzymes genes, such as coagulase was absent.
Table 1. The oligonucleotide primers used in PCR assay for virulence genes detection in TYH1 and TYH1 ΔhdcA.

| PCR set  | Gene | Nucleotide sequence (5’-3’)                                                                 | Application size (bp) |
|----------|------|---------------------------------------------------------------------------------------------|-----------------------|
| Multiplex a | Sea  | CTTTGAAAACGGTTAAAAACG<br>TCGAAACCTCCATGAAAAAC                                             | 127                   |
| Multiplex | Seb   | TCGCATCAACTTGACAAACG<br>GCAGGTACTCTATAAGTGCTGC                                           | 477                   |
| Multiplex | Sec   | CTCAGAAGACTAGCATAAAAGCTAGG<br>TCAAAAATCGGATTACACTATCC                                   | 271                   |
| Multiplex | Sed   | CTAATTTGTAAATCTCCTTAAAACG<br>TTATGCTATATCTTATAAGGTAAACATC                                | 318                   |
| Multiplex | see   | CAGTACCTATAGATAAGTTAAAAACAGG<br>TAACCTACGTTGGACCCCTTC                                      | 178                   |
| Uniplex b | selq  | AAATCTCTGGGTCAATGGTAAGC<br>TTGTATTCGGTTTTGTAGTATTTCG                                        | 122                   |
| Uniplex b | tta  | AAGCCCTTTTGTCTTGCG<br>ATCGAATCTGGGCCATCTT                                               | 447                   |
| Uniplex c | Coa   | CGAGACCAAGATTCAAAAAG<br>AAAGAAAACCACACTCATCA                                              | Variable              |
| Uniplex d | Nuc   | GCGATTGATGGTGATACCTTT<br>AGCCAAGCCTTGGACGAACCTAAAGC                                         | 270-300               |

**Fig. 1.** PCR validation of TYH1 recombinant strains. M: 100 bp ladder; 1, 2: TYH1 ΔhdcA; 3: TYH1 WT; 4: PKOR1 containing 300 bp hdcA gene; 5: S. aureus RN4220 containing PKOR1.

**DISCUSSION**

Histamine is detected in various fermented food especially in fish and sea foods, which has adverse effect on health of its consumers. Therefore, an importance arises to inhibit or decrease the histamine accumulation in these food sources. Among the potential approaches, controlling the bacterial decarboxylase activity is more reliable than others.

In the present study, HdcA deletion mutant was more successful than the inhibition of histamine-producing bacteria and degradation activities of microorganisms for controlling the production of histamine. Therefore, controlling histamine-related enzymes activity, inhibition the enzyme activity or elimination of the gene can be introduced as more practical approaches than others (8, 17). However,
the lack of information about histamine producing genes in staphylococci is considerable. Interestingly, our results are similar to the study performed by Guo et al. (17), in which PEP4 gene in Saccharomyces cerevisiae was knocked out. The PEP4 gene encodes an enzyme capable of producing free amino acids. They observed that, this knockout strain exhibited less ability in production of biogenic amines. The PrA activity and the amino acid concentration of this mutant strain reduced remarkably when compared to the wild type strain. The main reason for these reductions was the low concentration of free amino acids. In the study conducted by Pashanghe et al. (8) histamine degrading activity of staphylococcal isolates was investigated. The highest histamine degrading activity was 58.33% related to isolate No. 605 identified as S. epidermidis. Also Zaman et al. (18) observed that S. carnosus FS19, isolated from fish sauce, was able to degrade 15.1% and 13.8% of histamine content. Due to the bacteria inability to degrade all the added histamine, Diamine oxidase (DAO) was examined as more promising method to degrade histamine (8). No studies had been conducted on eliminating histidine decarboxylase gene, which is the first and most effective agent in preventing histamine accumulation.

ΔhdcA did not harbor any of the superantigenic toxin genes. Similar result of virulence genes is also found in S. epidermidis strain RP62A (19). These findings can explain why S. epidermidis strains are common inhabitants of skin or mucous membrane, but usually do not invade deeper tissues. To the best of our knowledge, there are a few existent reports on the prevalence of virulence genes in S. epidermidis to make a comparison. Similar results of safety assessment of staphylococcal strains which is in accordance with our study reported by Zhang et al. (19), showing that compared to the pathogenicity factors in S. aureus, virulence genes were not found in S. epidermidis strain (ATCC 12228). Interestingly, Rahmel et al. (15) observed that S. epidermidis 4S09 did not show any of the five classical enterotoxin genes. In this study ΔhdcA did not show a prevalence of superantigenic toxin and coa genes. Despite the presence of nuc gene, existence of the genes does not indicate their expression and toxin production. From the opposed point of view, the absence of virulence genes is a main criterion for selection of strains as potential starter cultures supplied by the strain TYH1.
CONCLUSION

In conclusion, eliminating of hdcA reduced the histamine formation activity in TYH1, and decreased the release of histamine. The finding of this research indicated that among the potential method of decreasing histamine content, generating the ΔhdcA mutant may be a reliable method for increasing the food safety of fish sauce and other fermented food products. Regarding the lack of information about histamine producing and degrading gene in staphylococcal strains, this information can be of great worth to the field of decreasing or inhibition of biogenic amines content in fermented foods.

REFERENCES

1. Tabanelli G. Biogenic Amines and Food Quality: Emerging challenges and public health concerns. Foods 2020; 9: 859.
2. Gonzaga VE, Lescano AG, Huamán AA, Salmón-Mulianovich G, Blazes DL. Histamine levels in fish from markets in Lima, Peru. J Food Prot 2009; 72: 1112-1115.
3. Lehane L, Olley J. Histamine fish poisoning revisited. Int J Food Microbiol 2000; 58: 1-37.
4. Linares DM, Martín MC, Ludero V, Alvarez MA, Fernández M. Biogenic amines in dairy products. Crit Rev Food Sci Nutr 2011; 51: 691-703.
5. Zaman MZ, Abu Bakar F, Jinap S, Bakar J. Novel starter cultures to inhibit biogenic amines accumulation during fish sauce fermentation. Int J Food Microbiol 2011; 145: 84-91.
6. Nala A, Flint S, Fletcher G, Bremer P, Meerdink G. Control of biogenic amines in food-existing and emerging approaches. J Food Sci 2010; 75: R139-150.
7. Mah JH, Hwang HJ. Inhibition of biogenic amine formation in a salted and fermented anchovy by Staphylococcus xylosus as a protective culture. Food Control 2009; 20: 796-801.
8. Pashanghe S, Shekarforoush SS, Aminlari M, Hosseinizadeh S, Nizet V, Dahesh S, et al. Inhibition of histamine accumulation by novel histamine degrading species of Staphylococcus sp. isolated from goats and sheep milk. Food Sci Natr 2022; 10: 354-362.
9. Yokoi KJ, Harada Y, Shoenzki KI, Satomi M, Taketo A, Kodaira KI. Characterization of the histidine decarboxylase gene of Staphylococcus epidermidis TYH1 coded on the staphylococcal cassette chromosome. Gene 2011; 477: 32-41.
10. Bae T, Schneewind O. Allelic replacement in Staphylococcus aureus with inducible counter-selection. Plasmid 2006; 55: 58-63.
11. Vitali L, Valese AC, Azevedo MS, Gonzaga LV, Costa ACO, Piovezan M, et al. Development of a fast and selective separation method to determine histamine in tuna fish samples using capillary zone electrophoresis. Talanta 2013; 106: 181-185.
12. Morandi S, Brasca M, Lodi R, Cremonesi P, Castiglioni B. Detection of classical enterotoxins and identification of enterotoxin genes in Staphylococcus aureus from milk and dairy products. Vet Microbiol 2007; 124: 66-72.
13. Javid F, Taku A, Bhat MA, Badroo GA, Mudasar M, Sofi TA. Molecular typing of Staphylococcus aureus based on coagulase gene. Vet World 2018; 11: 423-430.
14. González-Domínguez MS, Carvajal HD, Calle-Echeverri DA, Chinchilla-Cárdenas D. Molecular detection and characterization of the mecA and nuc genes from Staphylococcus species (S. aureus, S. pseudintermedius, and S. schleiferi) isolated from dogs suffering superficial pyoderma and their antimicrobial resistance profiles. Front Vet Sci 2020; 7: 376.
15. Rahmel S, Hosseinizadeh S, Shekarforoush SS, Torriani S, Gatto V, Pashanghe S. Safety hazards in bacteriocinogenic Staphylococcus strains isolated from goat and sheep milk. Microb Pathog 2018; 116: 100-108.
16. Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in Staphylococcus aureus isolates. FEMS Microbiol Lett 2005; 246: 191-198.
17. Guo X, Guan X, Wang Y, Li L, Wu D, Chen Y, et al. Reduction of biogenic amines production by eliminating the PEP4 gene in Saccharomyces cerevisiae during fermentation of Chinese rice wine. Food Chem 2015; 178: 208-211.
18. Zaman MZ, Abu Bakar F, Selamat J, Bakar J, See Ang S, Chong CY. Degradation of histamine by the halotolerant Staphylococcus carnosus FS19 isolate obtained from fish sauce. Food Control 2014; 40: 58-63.
19. Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, et al. Genome-based analysis of virulence genes in a non-biofilm-forming Staphylococcus epidermidis strain (ATCC 12228). Mol Microbiol 2003; 49: 1577-1593.