บทความทบทวนวรรณกรรมความเสถียรภาพและความคงทนต่อ pH ของสารพิษจากเชื้อโรคที่สำคัญในอาหารและการประยุกต์ใช้องค์ความรู้

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บทคัดย่อ

สารพิษเป็นสาเหตุสำคัญของการเจ็บป่วยจากอาหารและการระบาดของโรคอาหารเป็นพิษในประเทศไทย โดยมากสารพิษที่สัมผัสในชีวิตประจำวันมีแหล่งกำเนิดจากจุลินทรีย์ เช่น staphylococcal enterotoxins, cereulide จาก *Bacillus cereus* และ enterotoxins ที่ก่อให้เกิดโรคอุจจาระร่วง สารพิษโบทูลินัม และenterotoxins ทั้งที่คงทนต่อความร้อนและไม่คงทนต่อความร้อนจากเชื้อ enterotoxigenic *Escherichia. coli* (ETEC) สารพิษจะต่างกันในเสถียรภาพและความคงทนต่อ pH และสภาวะความร้อนต่าง ๆ เนื่องจากความผันแปรของโครงสร้างโมเลกุลและคุณสมบัติทางชีวเคมี แม้ว่าสารพิษบางชนิดจะแตกตัวในสภาวะกรดหรือด่างอ่อน ๆ หรือเมื่อมีการใช้ความร้อนที่ไม่รุนแรง สารพิษชนิดอื่น ๆ จะยังเสถียรภาพในสภาวะที่เป็นกรดและความร้อนสูงเช่น *Aspergillus* aflatoxin และ tetrodotoxin ที่พบในปลาปักเป้า ด้วยเหตุนี้จึงมีการใช้เทคนิคหลายอย่างในอุตสาหกรรมอาหารเพื่อขจัดสารพิษและจุลินทรีย์ที่เป็นอันตราย โดยวิธีการทั่วไปคือการใช้ความร้อนสูง ในขณะที่วิธีการใหม่ ๆ ได้แก่ ammoniation การลดความเป็นพิษโดยวิธีทางชีวภาพ การใช้ความดันไฮโดร-สแตติกสูง (HHP) และ กระบวนการ pulsed light system ยังคงอยู่ในขั้นตอนการศึกษาและประเมินประสิทธิภาพ อย่างไรก็ตามพบว่า มีการศึกษาการใช้เทคโนโลยีใหม่ ๆ ในการยับยั้งสารพิษน้อยมากเนื่องจากการศึกษาส่วนใหญ่ศึกษาผลกระทบต่อเซลล์

คำสำคัญ : สารพิษ การยับยั้งสารพิษ ความเสถียรภาพของสารพิษ จุลินทรีย์ก่อโรคในอาหาร

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A Review of Thermal and pH Stability of Important Foodborne Pathogen Toxins and Their Applications

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Abstract

Toxins are the major cause of foodborne illness and food poisoning outbreaks in Thailand. Those that we often come into contact on a daily basis are of microbial origin, such as staphylococcal enterotoxins (SEs), *Bacillus cereus* cereulide and diarrhoeal enterotoxins, botulinum neurotoxin, heat stable enterotoxins (STs), and heat labile enterotoxins (LTs) of enterotoxigenic *Escherichia. coli* (ETEC). Due to variation in molecular structure and biochemical properties, toxins differ in their stability and resistance towards various pH and heating conditions. While some toxins denature in slightly acidic or basic conditions, or with mild heat application, other types remain stable in moderately acidic and heat sterilization conditions, such as the *Aspergillus* aflatoxin and tetrodotoxin commonly found in puffer fish. Many techniques have been implemented in the food industry to eradicate hazardous microorganisms and their toxins. Common methodologies involve high heat processing (thermal inactivation), while novel methods, including ammoniation, biological detoxification, non-thermal high hydrostatic pressure (HHP), and pulsed light system (PLS) processing are currently being studied in terms of effectiveness. However, little is known of the application of novel technologies to inactivate toxins as most studies investigated the effects on vegetative cells.

Keywords: Toxins, Toxin inactivation, Toxin stability, Foodborne pathogen

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Food poisoning and foodborne illness in Thailand

Thailand is one of the largest food exporters of Asia. Many food products of Thailand, including rice, seafood, livestock, fruit and vegetable products, seasonings, and ingredients contribute to the country's socio-economic wellbeing. Thailand's value of food product exports has increased by 4.45% in 2018 compared to the previous year, and it is currently regarded as a strategic hub for global food production[1]. Thus, food poisoning and foodborne illness is a major issue of health concern[2]. Even though toxins can be derived from bacteria, fungal, algae, plant, and animal sources such as mycotoxins (e.g., aflatoxin, ochratoxin, citrinin, ergot, patulin, and fusarial toxins), saxitoxins (STXs) of microalgae, and tetrodotoxins (TTXs) of fish and shellfish poisoning, natural plant toxins (e.g., cyanogenic glycosides in bamboo shoots and cassava roots, glycoalkaloids in potatoes, and muscarine in wild mushrooms), those that we potentially come into contact on a daily basis are of bacterial origins due to food consumption[3-5]. *Staphylococcus aureus*, *Bacillus cereus,* listeria, salmonella, *Escherichia coli*, *Clostridium botulinum*, and *Pseudomonas* spp. are the key producers of endotoxins and exotoxins in food. Staphylococcal food poisoning (SFP) is a common foodborne illness caused by ingestion of staphylococcal enterotoxins (SEs), which are globular single-chain proteins produced by enterotoxigenic strains of coagulase-positive staphylococci[6]. *B. cereus* is known to cause two types of gastrointestinal disease, diarrheal and emetic, caused by different types of toxins. The emetic type is caused by cereulide intoxication, while the diarrheal type is caused by the ingestion of vegetative cells capable of producing enterotoxins, such as the haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe), and cytotoxin K (CytK), in the small intestine[7]. Foodborne botulism is a neuroparalytic disease with high fatality rate. It frequently occurs following the ingestion of botulinum neurotoxin actively secreted by *C. botulinum*[8]. Some other toxins include listeriolysin O secreted by *Listeria monocytogenes*, Shiga toxin and Shiga-like toxin (verotoxin and verocytotoxin) produced by enterohemorrhagic strains of *E. coli* (EHEC), and extracellular cytotoxin and exotoxin A of *Pseudomonas aeruginosa*. These toxins differ in stability towards adverse environmental conditions, attributed to variations in molecular structure and biochemical characteristics. Upon exposure to elevated temperature or alteration in pH,

the toxin may be inactivated, rendering the inactivation information of interest, applicable in certain context such as the food industry.

Stability of toxin in various temperature conditions (heat labile versus heat stable)

Heat treatment is a processing method commonly used in the industry to inactivate bacterial spores and vegetative cells in food. Table 1 shows the stability of selected toxins in various thermal and pH conditions. Bacterial endotoxins are moderately heat stable lipopolysaccharides (LPS) found in the outer membrane of gram-negative bacteria, and secreted via lysis of bacterial cells. Exotoxins are proteins synthesized by gram-negative and gram-positive bacteria with specific toxicity and greater potency due to enzymatic action. Exotoxins, especially from *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*, are responsible for majority of the foodborne outbreak cases throughout the world[25]. Staphylococcal enterotoxins are a family of over 20 distinct proteinaceous toxins (SEA, SEB, …, SEO) structurally and functionally related by amino sequence homology[26]. Staphylococcal enterotoxins are heat resistant, but subjected to heat inactivation in the sterilization of canned foods. A study by Regenthal *et al*. (2017) found no observable changes to the secondary structure of SEH toxin upon heat treatment up to 95°C, while reversible denaturation occurred with the tertiary structure, suggesting that toxins secreted by *S. aureus* varied in their ability to withstand heat[27]. Other microbial toxins exhibit greater heat stability, where normal heat sterilization conditions such as in steam autoclave (121°C, 15-20 min.) are incapable of inactivating them. Examples include cereulide, an emetic toxin produced by *B. cereus* and aflatoxin. Aflatoxins are stable in high dry heat conditions due to high decomposition temperatures, thus temperatures above 150°C are required to acquire partial destruction of the toxins (M_P = 268-269°C). Furthermore, previous studies have shown that the *Aspergillus* aflatoxin B_1 commonly found in roasted peanuts was susceptible to degradation at heating temperatures 160°C and above, while ochratoxin A secreted by toxigenic *Aspergillus* and *Penicillium* remained stable up to 180°C[28]. Over 20 species of puffer fish harbor tetrodotoxin (TTX), a naturally-occurring water soluble nonprotein alkaloid neurotoxin known to demonstrate heat stability, especially in acidic environments. TTX structure remains intact under heat-applied cooking conditions, thus

conventional heating methods do not remove TTX toxicity but rather increase its potency[29]. Although thermal processing is incapable of inactivating some heat-stable exotoxins, proteolytic botulinum neurotoxins (BoNTs) are heat-labile and denature with appropriate thermal processing. In the food industry, the "botulinum cook" (121°C, 3 min) is applied in low acid canned food processing to inactivate *C. botulinum* spores[30]. Heat-labile enterotoxigenic *E. coli* (ETEC) enterotoxins (LTs) are completely inactivated after heating for 30 minutes at 60°C, while heat-stable enterotoxins (STs) remain active at 100°C after 15-30 minutes of exposure. *C. perfringens* enterotoxins are sensitive to heat, and can be inactivated by heating at 60°C for 5 minutes[16].

Stability of toxins in various pH (acidic, neutral, alkaline)

The structural integrity of proteinaceous toxins is affected by changes in pH condition. While staphylococcal enterotoxins, SEA and SEE in particular, showed greater stability in acidic environments, SEH remained more stable in neutral pH conditions[27]. Our study using a commercial detection system to detect staphylococcal enterotoxins A to E showed inactivation of *S. aureus* enterotoxins upon challenging the pickled cucumber fermentation process with various concentrations of *S. aureus* inoculum. Results indicated safe consumption of pickled cucumber due to low pH of the product (unpublished work).

Paralytic shellfish poisoning toxins, including saxitoxin (STX), are imidazoline derivatives often found accumulated in shellfish, such as mussels, clams, and scallops. These toxins are stable in acidic conditions of pH 3.0–4.0 regardless of the storage temperature conditions[31]. Likewise, tetrodotoxins (TTX) are stable when stored frozen in mildly acidic conditions (pH 4.0-5.0), resistant to boiling, but denature in strong acid (~pH 2.0) and basic conditions above pH 7.0[32]. On the other hand, *C. botulinum* type A neurotoxins (BoNT/A complex) responsible for infant botulism, wound botulism, and foodborne colonization of the adult intestine showed increased thermal stability with increased pH along the range of pH 3.0 to 8.0. The toxin demonstrated greatest stability at pH 4.0–5.0, and may be susceptible to complete destabilization at pH range of 6.0 to 8.0. However, the 150 kDa neurotoxin component (NT) of the BoNT/A complex is most stable at pH 5.0–7.0[13]. Past studies have shown that listeria, which are non-acid fast

rods, grow best along the pH range of 6.0–8.0, with no growth observed in acid conditions below pH 4.0. However, listeriolysin O (LLO) demonstrated hemolytic activity at pH 5.5, but not at pH 7.0[16]. The release of heat-labile toxins (LTs) by enterotoxigenic *E. coli* (EHEC) strains are maximized at pH 7.5–8.0. The toxins consist of five B subunits (LTB) and one catalytic A subunit (LTA), arranged into holotoxins with remarkable stability when reaching the periplasm[33]. The structure of holotoxin remains intact along the pH range of 2.0 to 11.0[17].

Table 1. Stability of toxins in various environmental conditions

Toxin	Foodborne pathogen / organism	Heat inactivation conditions	pH of optimal stability
Staphylococcal enterotoxin B	*Staphylococcus aureus*	149°C, 1 h. 40 min. (A_w 0.99)[9]	4.8-9[9]
Cereulide	*Bacillus cereus*	>121°C, >2 h.*[10]	2-11[11]
Diarrhoeal enterotoxins (Hbl, Nhe, CytK)		56°C, 5 min.[11]	4-11[11]
Botulinum neurotoxin	*Clostridium botulinum*	85°C, 1 min. 80°C, 6 min. 65°C, 1.5 h.[9]	4.2–5.5[9, 12]
Shiga toxin Stx2	*Escherichia coli*	100°C, 5 min.[13]	5-6[14]
Heat-labile enterotoxin	*Escherichia coli* *Vibrio cholera*	56°C, 20 min.[15] 60°C, 30 min.[16]	2-11[17]
Cholera toxin		100°C, 10 min.[15]	7-9[15]
Listeriolysin O	*Listeria monocytogenes*	≥100°C, 30 min.[18]	5.5-6[18]
Exotoxin A	*Pseudomonas aeruginosa*	70°C, 30 min.[19]	>5[20]
Aflatoxin	*Aspergillus*	237-306°C*[21]	3-10[22]
Tetrodotoxin	Tetraodontidae (puffer fish), Gobiidae, Amphibia, blue-ringed octopus, invertebrates	>260°C (dry heat), 10 min.*[23]	<8[24]
Saxitoxin	Dinoflagellates (algae)	>260°C (dry heat), 10 min.*[23]	<8[24]

*Normal heat sterilization conditions insufficient to destroy the toxin

Application of toxins inactivation in the food industry

Toxins are the major virulence factors known to initiate symptoms of food poisoning. Sources of toxins may vary from contaminants to the presence of pathogens in food due to improper sanitary measures applied during food processing and handling[34]. A study conducted by Ananchaipattana *et al*. (2012) examined the prevalence of foodborne pathogens in Thai retailed foods. It was shown that foods purchased from the supermarket contained moderately high levels of toxigenic pathogens, including *Salmonella* spp., *Listeria* spp., *Staphylococcus* spp., and *Bacillus cereus*[35]. Conventional heat processing methods, such as sterilization and pasteurization, have long been used in food processing to inactivate biological hazards in edible products, and is effective in reducing microbial load of meat, poultry, and dairy products. However, novel thermal technologies, such as ohmic, dielectric, radio-frequency (RF), and microwave heating, have recently become common in the food industry[36]. Yet, these methods may be insufficient to eradicate preformed bacterial toxins, such as the toxins of *Staphylococcus aureus*. Staphylococcal enterotoxins (SEs) are heat-stable and can tolerate low pH. According to Regenthal *et al.* (2017), while some SEs aggregate with persistent heating, some may develop secondary and tertiary structures that remain intact and reversible during cooling[27]. SEH toxins, the cause of major staphylococcal food poisoning outbreaks from neutral pH dairy products, was able to retain its tertiary structure under vigorous heat processing. At lower pH, SEA and SEE secondary structures were able to persist thermal treatment. Their study indicated the necessity of increasing pH of foods along with appropriate heating conditions[27].

Novel non-thermal methods of microbial inactivation and decontamination of food products include non-thermal pulsed-light system (PLS), pulsed electric field (PEF), and high hydrostatic pressure (HHP) processing. PLS involves decontamination of food upon exposure of intense, short-duration pulses of a broad spectrum of UV light to the surface of a food or UV-transparent food packaging. The process was shown to significantly reduce the number of bacteria (i.e. 6 log reductions in *L. monocytogenes*, *S. aureus*, *B. cereus*, and *E. coli* O157:H7 with 200 pulses of high-UV light), yeasts, and viruses, and to some degree fungi on solid surfaces. Though having lethal effects on vegetative pathogens on a genomic level,

there are some limitations to the technology. Generally, foods have irregular surfaces which allow different levels of exposure to microbial contaminants, hindering overall efficiency of microbial destruction. Furthermore, UV light has the ability to penetrate only ~2 μm thickness of non-transparent matrices, limiting the treatment merely applicable along the surface. Radiation treatment is not as effective for foods high in fat and protein, and a reduction in efficiency was also observed with increased *L. monocytogenes* and overall microbial populations in the food[37]. Proteins inhibit viral inactivation during the treatment, raising concerns of the presence of protein toxins and whether they are sufficiently inactivated with novel non-thermal treatment methods[38]. PEF delivers high voltage electrical pulses (20-80 kV cm^{-1}), which initiates the disruption of microbial cell membranes, and enzyme inactivation with combined effects of mild heat in pumpable fluid and semi-fluid foods. However, there is currently insufficient information on its ability in inactivating food toxins. Thus, further study of toxin inactivation by novel non-thermal methods is of interest.

HHP inactivates vegetative microorganisms and enzymes with pressure instead of heat, and is a processing technology of major research interest[37]. A study by Margosch *et al*. (2005) examined the combined effect of pressure (0.1–800 MPa) and heat (5–121°C) treatment on bacterial enterotoxins of *Vibrio cholerae*, *S. aureus*, *B. cereus*, and *E. coli*. Results revealed synergistic effects of pressurization and heat treatment on the degree of toxins inactivation, suggesting possible equivalence in effectiveness at lower temperature and reduced incubation time. Significant reductions in the immuno-activity and structural disintegrations of SEs and cholera toxins (CTs) in enzyme immunoassays (EIA) with combined pressurization at 800 MPa and ≥80°C heat treatment were observed. Applications of high hydrostatic pressure food treatment are still being developed due to it having lesser undesirable effects on sensory and nutritional quality as compared to conventional heating. Although there is sufficient evidence supporting HHP inactivation of vegetative microorganisms and denaturation of protein, little information is currently available regarding stability of bacterial toxins towards the treatment[39].

Many strategies of mycotoxin detoxification were proposed and implemented within the food industry. Mycotoxins, such as aflatoxin M_1, have demonstrated resistance toward all forms of

available thermal treatment. Chemical inactivation methods include ammoniation of peanut, corn, and cereal grain, and treatment of raw food materials with chlorine dioxide for trichothecene mycotoxin inactivation or citric acid for aflatoxin B_1 and aflatoxin B_2 inactivation in maize grains, sulfhydryl compounds, and feed additives[40]. A study by Trujillo and Yepez (2003) demonstrated effectiveness and permanence of ammoniation process on inactivation of aflatoxin B_1 in rice grains through combined high pressure/high temperature (HP/HT) and atmospheric temperature/moderate temperature (AT/MT) conditions with higher temperatures and longer processing time[41]. Biological detoxification may occur naturally in fungal strains capable of degrading or transforming their mycotoxins in response to change in environmental conditions, via pure culture inoculation of detoxifying strains of bacteria and fungi, or via insertion of genes encoding for detoxification activities in the host. Common physical methods include extrusion cooking, UV radiation, gamma radiation, and HHP. However, the extrusion method was able to achieve only partial inactivation of aflatoxin B_1 in corn flour, and 76% reduction in aflatoxins following extreme processing conditions at 160°C in cottonseed. Aflatoxin B_1 activity in peanut meal was reduced by 100% following exposure to 10 kg gamma ray, while aflatoxin M_1 activity in milk was reduced by 3.6-100% with increased time of exposure to UV light[40].

Conclusion

Microbial toxins are major virulence factors of foodborne illness in Thailand, varying in heat and pH stability. While some toxins are inactivated via mild heat exposure, some other resistant types can still retain their structural integrity under conditions as severe as thermal sterilization, or extremely acidic and basic environments. Toxins may be ingested directly or later secreted by microorganisms invading the gut microbiota sourced from contaminated food. Improper sanitation of food contact surfaces, poor maintenance of food hygiene, and food handling are some of the leading factors of toxin prevalence in food. It is crucial for food service providers to carry out proper sanitary measures to ensure minimized risks towards contamination. A number of food and food contact surface sterilization methods are currently available and applied within the industry. It is recommended that sterilization techniques shall be selected based on suitability, efficiency in achieving sterility, and overall effectiveness in application to

avoid contamination and minimize the occurrence of toxin-mediated hazards.

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