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Bacteriology of Indole Production in Shrimp Homogenates Held at Different Temperatures

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ABSTRACT

Homogenized, head-on, white shrimp (*Penaeus setiferus*) were held at 4, 12 and 22°C until putrefactive spoilage occurred. Repetitive bacterial sampling was performed and 1647 bacterial isolations were made from the shrimp homogenates. Of these, 42 isolates (2.6%) produced indole. Isolates that produced indole belonged to the genera *Flavobacterium* (52.4%), *Aeromonas* (23.8%), *Proteus* (21.4%) and *Yersinia* (2.5%). No *Escherichia coli* were isolated. *Aeromonas* and *Proteus* exhibited proteolysis and were able to produce indole in shrimp extracts without added L-tryptophan. These organisms favored higher growth temperatures. The majority of the *Flavobacterium* isolates were psychrotrophic, non-proteolytic and could not produce indole in shrimp extracts without added L-tryptophan. Suppression of bacterial reproduction with antibacterial compounds inhibited indole production. Two paths of indole production are suggested based on temperature of decomposition.

Due to lack of proper handling and processing in the country of origin, spoiled or decomposed shrimp are often encountered in shipments entering domestic ports. To ensure product safety and quality, the U.S. Food and Drug Administration (FDA) constantly evaluated imported shrimp for *Salmonella,* filth and decomposition. Although determined subjectively by sensory evaluation, decomposition is substantiated objectively, when needed, by determining the concentration of indole in the product (*7*).

In a previous report (*3*), we established that temperature of storage was the primary factor affecting the formation of indole in raw shrimp. Even though indole was also present in shrimp stored over prolonged time periods on ice or refrigerated, the results in that report suggested that indole formation is most likely related to high temperature abuse, presumably because the ability to convert tryptophan to indole is common among mesophilic microorganisms. This is in agreement with the results of Ponder (*6*) who suggested that organisms such as *Proteus morganii* or *Escherichia coli* were major indole producers in decomposing shrimp. However, since low levels of indole were also encountered in iced or refrigerated shrimp, psychrotrophic organisms or endogenous enzyme systems may play a role in the conversion of L-tryptophan to indole. The purpose of this study was to elucidate the mechanism of indole formation during spoilage of common shrimp harvested from the Gulf of Mexico. Specific objectives were to: (a) isolate and identify indole-producing microorganisms from shrimp homogenates undergoing spoilage under different conditions, (b) demonstrate the potential of these organisms to produce indole in sterile shrimp extracts, (c) determine the temperature tolerance of the indole-producing organisms, and (d) determine if indole can be produced by endogenous shrimp enzyme systems.

MATERIALS AND METHODS

Raw material and bacterial isolates

White shrimp (*Penaeus setiferus*), of size equivalent to approximately 40 tails per pound, were obtained from commercial shrimp trawlers operating along the Texas coast. Extreme care was taken to minimize external contamination when the shrimp were separated from the incidental catch, packed on ice and transported to the Seafood Technology Laboratory at Texas A&M University in College Station.

Upon arrival, the shrimp were divided in six lots each weighing ca. 1.8 kg. Each lot (head-on) was homogenized with two parts of sterile distilled water using a sterile stainless steel blender. The shrimp were homogenized to achieve even distribution of the different additives and to determine if endogenous indole-producing enzymes may be associated with the gut content or digestive system. L-tryptophan (Sigma Chemical Co., St. Louis, MO) was added to three of six lots at a concentration of 10 mg/100 g shrimp at the time of homogenization. Lots were paired as shrimp only and shrimp plus added tryptophan and stored at 4, 12 or 22°C until severely decomposed.

The homogenates were initially sampled at the time of homogenization then at 3-h intervals at 4 and 12°C. Bacterial numbers were determined by the spread-plate method using tryptic soy agar (TSA, Difco). Appropriate dilutions using 0.1% peptone water in 0.1-ml quantities were spread evenly over the surface of prepoured agar plates. Plates were incubated at 22°C for 48 h for samples from all of the homogenates, and additionally at 12°C for 4 d and 4°C for 7 d for samples stored at those temperatures.

After the plates were counted, representative colony types appearing on countable plates (30 to 300 colonies) were picked and transferred

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to tryptic soy broth (TSB, Difco) and incubated at the original temperatures of isolation. The broth cultures were tested for indole according to the method of Vracko and Sherris (*9*). Indole-positive organisms were stored on TSA slants at their temperature of isolation until further testing. After the organisms had been transferred to fresh TSA slants and stored for an additional 48 h, standard biochemical tests and protocols were used to identify each indole-positive organism to the genus and, when practical, species level (*1,2,5,8*).

Minimum growth temperature

Each indole-positive organism was transferred to tubes containing TSA and incubated at 4 and 8°C for 7 d, 12 and 16°C for 4 d, and 22°C for 2 d. Active growth was determined spectrophotometrically by measuring light transmission (% T) at 655 nm. Tubes exhibiting ≥90% T were regarded as negative, whereas tubes with light transmission < 90% T were considered positive. For a final determination, the organisms were reinoculated and incubated at 2°C lower than the established initial minimum growth temperature. Incubation period for the reinoculated cultures were: 10 d for 2 and 6°C, 4 d for 10, 14 and 18°C, and 2 d at 20°C. At the end of the incubation period, each inoculum was tested for indole production as described above.

Proteolytic activity

Standard methods caseinate agar (SMCA) was prepared according to the method of the American Public Health Association (*1*) to determine the proteolytic activity of the indole-positive organisms. SMCA plates were inoculated with the indole-positive organisms and incubated at the temperature of original isolation. Proteolytic activity was determined on the basis of precipitation or clearing of the medium.

Sterile shrimp extracts

Shrimp homogenates (prepared as described above) were kept at -30°C for 5 d, tempered for 18 h at 4°C, homogenized and centrifuged at 20,000 × *g* for 1 h at 10°C. The homogenates were then filter-sterilized through a Gelman GA-6 0.45-μm membrane (Gelman Instruments Co.) and tested for sterility by the spread-plate method on TSA.

Indole-positive organisms were inoculated into 0.5 ml of sterile shrimp extracts with and without added L-tryptophan and incubated at the original temperature of isolation. Uninoculated controls both with and without L-tryptophan were incubated in parallel with the inoculated samples. At the end of the incubation period, each sample was tested for indole production as described above.

Endogenous enzymes

Homogenates prepared as described above were supplemented with 0.4% (wt/wt) dihydrostreptomycin (Sigma Chemical Co.), 1.0% (wt/wt) thimersal (ethylmercurithiosalicylate, Eastman Kodak Co.), and 0.4% (wt/wt) chloramphenicol (Sigma Chemical Co.) to determine if endogenous enzyme systems can play a role in the production of indole during storage of shrimp. Preliminary investigations demonstrated that this mixture caused dramatic reductions in total plate counts and inhibited microbial growth when added to shrimp homogenates. The antibiotic-treated homogenates were stored at 4, 12 and 22°C and were tested for both plate counts and indole after 0, 24, 48 and 96 h of incubation.

RESULTS AND DISCUSSION

Recovery and identification of indole-producing organisms

Of 1647 colonies selected from the various TSA plates, 42 (2.6%) were demonstrated to be indole-positive. Four of these were isolated from homogenates incubated at 4°C, 12 to 14°C and 22 to 24°C. As shown in Table 1, *Flavobacterium* spp. were the dominant organisms representing 52.4% of the total number of indole-positive isolates. This was followed by *Aeromonas* spp. representing 23.8%, *Proteus vulgaris, Proteus morganii,* and *Proteus inconstans* combined for a total of 21.4%, and finally an indole-positive *Yersinia* sp. was isolated representing 2.5% of the total number of indole-positive organisms. No *Escherichia coli* were detected in this study. This is in disagreement with Ponder (*6*) who indicated that *E. coli* is a major indole-producing organism associated with shrimp. There could be a number of reasons why *E. coli* was not detected during this work. Even at an incubation temperature of 22°C, this organism may not be able to successfully compete in incubated shrimp homogenates and was thus not recovered. Differences in location of harvest, natural microflora and handling procedures could also be factors responsible for the differences between the two studies. It is very likely that *E. coli* plays a major part in the production of indole in shrimp handled and processed under poor sanitary conditions.

Minimum temperature for growth and indole production

Minimum growth temperature and minimum temperatures for indole production of the indole-producing organisms are shown in Table 1. The lowest temperature tested was 2°C and the organisms reported growing at this temperature may have had the ability to grow at even lower temperatures. A wide range of minimum growth temperatures, from below 2°C to as high as 14°C, was observed with a large number of psychrotrophic organisms. With only few exceptions, *Flavobacterium* spp. were able to produce indole at the minimum temperature tested which was 2°C. However, for most of the other organisms this was not true. While able to grow at low temperatures, most of the organisms required higher temperatures for indole production. This was especially true for *Aeromonas* spp. where, with only one exception, 22°C was a requirement for indole production. If a ratio between mean minimum growth temperature and mean minimum temperature for indole production is used to characterize the ability of the different genera to produce indole at low temperatures, *Flavobacterium, Aeromonas,* and *Proteus* were 5.2/7.6, 5.8/20.4, and 6.6°C/14.5°C respectively. From these ratios, it is evident that while *Aeromonas* and *Proteus* spp. are, in most cases, able to grow at low temperatures, with few exceptions they require higher temperatures for indole production. Although a few *Flavobacterium* spp. also required high temperatures for indole production, most of the organisms within this genus could produce indole at the minimum growth temperature tested. It is also important to note that the original isolations of *Proteus* and *Aeromonas* were made predominately from homogenates stored at 22°C, whereas the indole-producing *Flavobacterium* spp. were recovered from homogenates held at all three incubation temperatures.

Proteolytic activity

Sixteen of the indole-producing organisms showed no visible proteolytic activity on standard casein agar (Table 1). This group included most of the *Flavobacterium* spp. together with *P. inconstans*. Limited proteolysis was

demonstrated by six organisms identified as *P. morganii*, *Aeromonas* spp., *Yersinia* sp., and one *Flavobacterium* sp. Five of the isolates, including four *Flavobacterium* spp. and one *P. morganii*, showed moderate proteolysis, whereas 13 isolates identified as *P. vulgaris*, *Aeromonas* spp., and *Flavobacterium* spp. had strong proteolytic activity. Two of the isolates did not grow on standard casein agar, hence their proteolytic activity could not be evaluated.

Although the ability of the proteolytic-positive organisms to breakdown milk proteins may not be directly related to their ability to digest shrimp proteins, the results from this study clearly indicate the active proteolytic activity of most of the *Flavobacterium* spp. There also appeared to be an indirect relationship between psychrotrophic properties and proteolytic ability in most of the indole-positive organisms. For example, the strongly proteolytic *Flavobacterium* spp. (isolates 30F, 31F and 32F)

TABLE 1. *Temperature, indole-producing and proteolytic characteristics of indole-positive organisms isolated from shrimp homogenates.*

Isolate[a]	Minimum growth temp. (°C)	Minimum temp. for indole prod. (°C)	Isolation temp. (°C)	Proteolytic activity[b]	Indole shrimp extract	Indole shrimp + tryptophan
4F	12	12	12	-	-	-
11F	2	6	4	-	-	+
12F	6	6	22	+ +	-	+
13F	6	6	22	-	-	-
16F	6	8	22	+ +	-	+
17F	4	4	22	-	-	-
26F	2	8	12	-	-	+
27F	2	4	12	-	-	+
28F	6	6	4	-	-	-
30F	2	22	22	+ + +	-	+
31F	14	16	22	+ + +	-	+
32F	8	22	12	+ + +	-	+
33F	14	14	4	+ +	-	+
34F	6	6	4	+ +	-	-
35F	12	12	12	NG	-	+
36F	2	2	12	-	-	+
37F	2	2	12	-	-	+
38F	2	2	12	-	-	+
39F	2	2	12	-	-	+
40F	2	2	12	-	-	+
41F	2	2	12	+	-	+
42F	2	2	12	-	-	+
6A	6	22	22	+ + +	+	+
7A	6	6	22	+ + +	+	+
8A	2	22	22	+ + +	+	+
15A	6	22	22	+ + +	+	+
19A	6	22	22	+ + +	+	+
20A	6	22	22	+ + +	+	+
21A	6	22	12	+	+	+
22A	6	22	22	+	+	+
23A	12	22	22	NG	-	-
29A	2	22	22	+ + +	+	+
1P	8	22	22	+ + +	+	+
2P	8	22	22	+ + +	+	+
3P	6	16	12	+ + +	+	+
5P	6	6	22	-	-	+
9P	4	8	22	-	-	+
10P	6	6	22	-	-	+
14P	8	22	22	+ +	+	+
18P	8	8	22	+	+	+
25P	6	22	22	+	+	+
24Y	4	22	22	+	+	+

[a]F, *Flavobacterium;* A, *Aeromonas;* P, *Proteus;* and Y, *Yersinia.*

[b], no precipitation and no clearing; +, precipitation and no clearing; + +, precipitation and clearing; + + +, total clearing; and NG, no growth.

were only able to produce indole at high temperatures, even though at least one of the isolates grew well at 2°C. Conversely, the non-proteolytic *Proteus* isolates (5P, 9P and 10P) were the organisms of that genus that produced indole at low temperatures.

Indole production in sterile shrimp extracts

Sixteen indole-producing organisms were also able to produce indole in sterile shrimp extracts when incubated at the original isolation temperature (Table 1). These isolates included *P. vulgaris, P. morganii, Aeromonas* spp., and one *Yersinia* sp. Twenty additional isolates were capable of producing indole in sterile shrimp extracts when L-tryptophan was added. This group consisted predominantly of *Flavobacterium* spp. Six of the isolates were unable to produce indole in either plain sterile extracts or sterile extracts supplemented with free L-tryptophan. Indole could not be detected in uninoculated sterile extracts.

All the organisms that produced indole in sterile extracts without added tryptophan were also proteolytic in nature. Since Cobb and Vanderzant (*4*) reported that tryptophan could not be detected in measurable amounts in deproteinated shrimp extracts, proteolytic activity may be a requirement in order to produce free tryptophan as substrate for indole formation. However, this does not explain why seven proteolytic *Flavobacterium* spp. were unable to produce indole in shrimp extracts without added tryptophan.

Endogenous enzymes

The combination of antibacterial compounds prevented the formation of indole in shrimp homogenates stored at 4, 12 and 22°C for 96, 48, and 24 h, respectively. Control homogenates without added antibiotics reached counts of approximately 10^{10} organisms/g at the end of the same storage period. The controls stored at 12 and 22°C were also positive for indole. The lack of indole production in homogenates treated with antibacterial compounds and production of indole in non-treated controls indicate the absence of indole production of endogenous tissue or gut enzymes. Indole production in shrimp tissue therefore appears to be related solely to bacterial action.

CONCLUSIONS

Although the microbial ecology in homogenated whole shrimp extracts is no doubt different from whole shrimp or shrimp tails, extracts were used throughout this study in order to test for the presence of endogenous indole-producing enzymes associated with the digestive system and also to achieve proper mixing of antibiotics and reaction substrates.

In general, this study has shown that indole production in shrimp is the result of bacterial action and that endogenous shrimp enzymes do not have the ability to convert tryptophan to indole. It also points out the importance of temperature in controlling the amount of indole produced during storage of fresh shrimp.

Of more significance, the study clearly reveals two distinctly different modes of indole production in shrimp, i.e., high temperature and low temperature. At temperatures above approximately 10°C, highly proteolytic indole-positive organisms, such as *Aeromonas* and *Proteus*, breakdown shrimp tissue proteins providing the tryptophan which is subsequently converted to indole. High levels of indole are therefore useful as indicators of temperature abuse during either harvesting, handling, processing and distribution.

Indole production in shrimp held at low temperatures is most likely due to psychrotrophic, non-proteolytic organisms, such as *Flavobacterium* spp., that comprise a very small portion of the bacterial population. Since they are nonproteolytic and shrimp tissue is void of free tryptophan, it is only after proteolytis by spoilage organisms, such as *Pseudomonas*, that low amounts of indole begin to appear in shrimp.

This research confirms earlier observations (*3*) that, while high indole levels in shrimp indicate decomposition through temperature abuse, decomposed shrimp held at low temperatures may not necessarily contain indole.

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