Research Note

A Rapid and Inexpensive Method for the Determination of Biogenic Amines from Bacterial Cultures by Thin-Layer Chromatography

EMILIA GARCÍA-MORUNO,† ALFONSO V. CARRASCOSA, AND ROSARIO MUÑOZ*

Departamento de Microbiología, Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas, Juan de la Cierva 3, 28006 Madrid, Spain

MS 04-414: Received 14 September 2004/Accepted 10 November 2004

ABSTRACT

This study describes a simple, rapid, and inexpensive method to determine the ability to produce biogenic amines (BA) by bacteria in liquid culture media containing the corresponding amino acid precursor. In view of their role as starters in food fermentation, BA formation by these microorganisms has to be taken into consideration by selecting appropriate strains. For the standardization of the assay pure BA were mixed. The method avoids a prior extraction step of the amines and allows the separation and identification of the amines histamine, tyramine, putrescine, and phenylethylamine using thin-layer chromatography. The method was successfully applied to several BA-producer bacterial strains. This method constitutes a simple solution to the previous reports describing false-positive reactions in routine screening procedures generally involving the use of a differential medium containing a pH indicator.

Biogenic amines (BA) often have been associated with food intoxications over the past 20 years. Therefore, certain BA could be used as an indicator for food quality and hygiene during food processing. Biologically active amines, such as histamine (HIS), tyramine (TYR), putrescine (PUT), and phenylethylamine (PEA), have been implicated in food poisoning incidents, usually from the consumption of fermented foods (24). Biogenic amines in foods are mainly formed by decarboxylation of the corresponding amino acids by microorganisms. The capability of BA formation has been described for several groups of microorganisms, mainly Enterobacteriaceae, Pseudomonas spp., enterococci, and some other lactic acid bacteria (8, 16, 17, 26). In the case of lactic acid bacteria, its amino acid decarboxylation activities should be tested prior its use as fermentation starter cultures.

Several qualitative and quantitative methods to determine the production of BA by microorganisms have been described. Decarboxylase activity is a conventional biochemical test for characterization and identification of microorganisms (5). Most microbiological screening procedures generally involve the use of a differential medium containing a pH indicator (2, 13, 14). However, some reports have described false-positive reactions in some of these media due to the formation of other alkaline compounds (1, 16). Nowadays, the histamine produced in food substrates is measured chiefly by means of high-pressure liquid chromatography (28), gas liquid chromatography (25), gel chromatography (20), and thin-layer chromatography (TLC) (1, 3, 10, 21, 22). Enzymatic methods including radioimmunoassays and enzyme-linked immunosorbent assay system have been applied to detect histamine (6, 12). Amino acid decarboxylase activities of Salmonella spp. and other Enterobacteriaceae were studied using the BioSys-128 (MicroSys, Inc., Ann Arbor, Mich.), an automated system for detection of microbial metabolic activities (23). Recently, an enzyme sensor array (9) and a capillary electrophoresis with laser-induced fluorescence detection method (11) have been described. All the aforementioned techniques, with the exception of TLC, require costly and sophisticated equipment, specially trained staff, and lengthy preparation of the extract of the sample to be analyzed. Therefore, small microbiology laboratories where this equipment is not available need a simple, rapid, and inexpensive method to determine BA produced by bacteria.

The objective of this study is to develop a simple and rapid assay suitable for application in small microbiology laboratories for the separation and identification of dansylated amines (i.e., HIS, TYR, PUT, and PEA) in bacterial cultures.

MATERIALS AND METHODS

Reagents. The amino acids (L-histidine monohydrochloride, L-tyrosine disodium salt, L-ornithine hydrochloride, and L-phenylalanine) and amines (histamine dihydrochloride, tyramine hydrochloride, putrescine dihydrochloride, β-phenylethylamine hydrochloride) were purchased from Sigma-Aldrich (Steinheim, Germany). Dansyl chloride was also purchased from Sigma-Aldrich. Chloroform, triethylamine, isopropanol, triethanolamine, and...
TLC plates (20 by 20 aluminum sheets precoated with 0.20 mm silica gel 60 F254) were purchased from Merck (Hohenbrunn, Germany).

**Biological strains and culture conditions.** Tyramine-producing *Lactobacillus brevis* CECT 5354 (ATCC 367) (15) was provided by the Spanish Type Culture Collection (CECT). *Lactobacillus* 30a, a histamine- and putrescine-producing strain (7, 27), was purchased from the American Type Culture Collection (ATCC). *Serratia liquefaciens* IFI-65, a putrescine-producing strain, was isolated from a meat product and belongs to the meat bacterial collection from the Instituto de Fermentaciones Industriales (IFI). *ENTEROCOCCUS FAECIUM* BIFI-58, a tyramine- and phenylethylamine-producing strain, was originally isolated from a grape must and belongs to the oenological bacterial collection from the Instituto de Fermentaciones Industriales (BIFI).

Bacterial strains were grown in commercial culture broth media supplemented with 0.5% of the corresponding amino acid precursor. In some analyses, bacteria producing two different amines were supplemented with both amino acid precursors. Lactobacilli and *E. faecium* BIFI-58 were grown in deMan Rogosa Sharpe (MRS) broth (4) (Difco, Becton Dickinson, Sparks, Md.) at 30°C during 7 days and *S. liquefaciens* IFI-65 in nutrient broth (Difco, Becton Dickinson) at 30°C with shaking during 3 days.

**Biogenic amine TLC analysis.** A stock standard solution of each amine (HIS, TYR, PUT, and PEA) was made by preparing a 2% solution in 40% ethanol. Different dilutions of this solution formed the working standard.

Amines were converted to their fluorescent dansyl derivatives using a modification of the method of Rosier and Van Peteghem (19) as follows, one volume of 250 mM disodium phosphate (pH 9.0) was added to one volume of the standard amino solution, 0.1 volume of 4 N sodium hydroxide solution, and two volumes of dansyl chloride solution (5 mg/ml of dansyl chloride in acetone). The reaction mixture was thoroughly mixed and then incubated in the dark at 55°C for 1 h. Then the samples were cooled and stored at 4°C until use.

The fractionation and detection of the amines were performed by modifying the method used by Shakila et al. (21) for the determination of biogenic amines in fish. The amines were fractionated on precoated silica gel 60 F254 TLC plates. Amine derivative extracts (10 μl) were applied 2 cm from the base of the plates with capillary pipettes. The dansylated compounds were separated by ascending development for 17 cm in chloroform-triethanolamine (4:1), a solvent mixture that was able to efficiently separate the four amine derivatives. Dansyl amino acids and ammonia showed lower mobility, remained near the origin, and did not interfere with the separation of amines.

After chromatography was complete, the plate was sprayed with isopropanol-triethanolamine (8:2) to enhance the fluorescence. The fluorescent dansyl derivative spots were visualized with the aid of a transluminator (Vilber Lourmat, Marne-la-Vallée, France) with a suitable UV-light source (312 nm). To enhance the resolution of the TLC method, a Digi Doc Photo Documentation System (Bio-Rad, Hercules, Calif.) was used. This system is dedicated to the acquisition of images and includes a digital Kodak DC265 photo camera. To enhance the TLC resolution we set this digital photo camera on a hood light proof that was covered with aluminum foil to increase the UV light signal by reflecting the UV light emitted by the transluminator located under the TLC plate.

TLC assays were performed in triplicate in order to test the repeatability of the method. This is a qualitative method; therefore, its repeatability could not be expressed as a numeric value. However, the TLC method showed a high repeatability since in all the TLC assays performed, the BA produced by the bacterial cultures presented the same Rf values and no cases of false-positive results were detected.

**Analysis of biogenic amines from bacterial cultures.** Bacterial strains were grown as described in the “Biological strains and culture conditions” section. After incubation, the broth media were centrifuged and the supernatants were analyzed for BA content. Analysis of amines produced by bacterial strains was performed directly from bacterial supernatants as described above. As compared with other described methods (1), this improved method did not require a prior extraction step of amines from the bacterial supernatants.

**RESULTS AND DISCUSSION**

TLC is a relatively cheap but powerful technique to screen microbial extracts for the presence of different types of amines. Extracts are spotted onto layers of cellulose or silica gel that are attached to glass plates or aluminum sheets. The dansyl derivatives of amines could be easily detected under UV light due to their fluorescent characteristics.

In order to determine the minimum detection level of the method for each one of the four amines analyzed (HIS, TYR, PUT, and PEA), different concentrations of the standard amines were tested on the TLC. The concentrations assayed ranged from 10,000 mg/liter to 10 mg/liter. Figure 1 showed the detection level for each amine tested. It can be observed that the method showed less sensitivity to HIS since 1,000 mg/liter is the lower concentration of HIS detected, while the other amines assayed were detected at 10 mg/liter level. Since the sample volume was 10 μl, 100 ng was the amine amount detected for TYR, PUT, and PEA. It has been previously shown (3) that from the amines analyzed, HIS showed the lowest recovery rates throughout the procedure, including extraction, derivatization, and thinline chromatography. However the detection level of HIS in the TLC method described in this study is adequate to detect HIS production when the bacteria is growing in a culture media supplemented with histidine where HIS levels higher than 6,000 mg/liter had been described (18).

It also could be observed that HIS is the amine that showed several tails running along the TLC. TYR and PEA only showed one tail, and in the case of TYR it disappears at the lower concentrations tested. PUT spots were symmetrically shaped and did not tail. Several biogenic amines often occur simultaneously in bacterial extracts, so that analytical procedures that permit the separation and estimation of these compounds in the same aliquot of an extract are desirable. In this regard, to check the availability of the method to detect simultaneously the four BA tested, a mixture of the amines was prepared. Based on the sensitivity showed by the method for each amine, the mixture contained HIS at 5,000 mg/liter, and TYR, PUT, and PEA at 250 mg/liter. As shown in Figure 2, a satisfactory separation was obtained. It is clear that the dansylated biogenic amines were well separated and easily identified. These amine spots are markedly different than the tails located below the PUT spot. The Rf values, as well as the charac-
teristic fluorescent colors of the dansylated amines (Table 1), were useful in their identification. The order of separation of amines from the bottom of the TLC plate was PUT, HIS, TYR, and PEA.

It has been previously reported in other TLC systems that each amine showed a fluorescent characteristics color (3, 21, 22). In the TLC system described in this study all the amines presented a greenish blue color with exception of HIS (Table 1).

The main application of this TLC method in microbiology laboratories could be to detect the capability of bacterial strains to produce BA. Figure 3 shows the TLC separation of amines from bacterial cultures. First of all, we observe the patterns given for the growth media. MRS broth alone gives a more apparent spot in the TLC plate (Fig. 3A, lane 1 and Fig. 3C, lane 1) than the nutrient broth (Fig. 3B, lane 1). The bacteria also were grown in culture media supplemented with the corresponding precursor amino acid.

As shown in Figure 3A (lane 2), L. brevis produces TYR from the tyrosine present in the MRS broth; when the culture media was supplemented with 0.5% tyrosine, an increase in the TYR formed could be observed. Also, when the media is supplemented with tyrosine, the tail observed from the standard TYR solution appears (Fig. 3A, lane 4). In a similar way, S. liquefaciens IFI-65 produces PUT from the nutrient broth (Fig. 3B, lane 2), which increases when the media was supplemented with ornithine (Fig. 3B, lane 3). In both cases, it might be concluded that even though the culture media alone gives faint spots in the TLC plates, the amines produced as consequence of bacterial growth can be easily detected even without amino acid supplementation.

In order to check the usefulness of the TLC method to detect simultaneously two different amines produced by the same bacteria, E. faecium BIFI-58 and Lactobacillus 30a were tested. E. faecium growing in MRS media produced enough PEA and TYR to be clearly differentially detected (Fig. 3C, lane 2). When the media was supplemented with phenylalanine (Fig. 3C, lane 3), tyrosine (Fig. 3C, lane 4), or both amino acids (Fig. 3C, lane 5), an increase in the corresponding amine is clearly observed. The same behavior was observed with Lactobacillus 30a growing in MRS media supplemented with histidine (Fig. 3D, lane 2) and ornithine (Fig. 3D, lane 3).

Several BA-forming species are of importance in food processes. Some of the positive strains might be used as starter, without prior knowledge of their potential to form BA. Therefore, the lack of production of these compounds needs also to be confirmed for the microorganism generally regarded as safe.

From the aforementioned results it could be concluded that the TLC procedure used in this study is simple, rapid,

\[
\text{TABLE 1. } R_f \text{ values and fluorescent colors of the standard biogenic amines separated by TLC technique}
\]

<table>
<thead>
<tr>
<th>Amines</th>
<th>( R_f ) values(^a)</th>
<th>Fluorescent color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>0.458</td>
<td>Greenish blue</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.849</td>
<td>Orange-yellow</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.915</td>
<td>Greenish blue</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>0.953</td>
<td>Greenish blue</td>
</tr>
</tbody>
</table>

\(^a\) The \( R_f \) values are the mean of three TLC runs.
and sensitive for the separation and estimation of BA and that it should be applicable to bacterial suspensions. As compared with previously described TLC methods, the proposed method avoids a prior extraction step of the amines. The method only requires a photo camera and an UV transilluminator, nowadays common equipment in a microbiology laboratory. This method is more sensitive and specific—avoiding false-positive results—than a differential culture media containing a pH indicator (2, 13) and has the advantages of being low in cost, reliable, easy to use, and fast to perform as compared with existing methods, such as the high-pressure liquid chromatography methods, which require sophisticated instrumentation, careful maintenance, expensive solvents, accessories, and high operational skills. The method allows for easy identification of the dansyl derivatives of HIS, TYR, PUT, and PEA in contrast to more laborious methods. This method can be easily adopted by microbiology laboratories as a qualitative tool for the analysis of the ability to form BA by bacteria.

ACKNOWLEDGMENTS

This work was supported by grant 07G/0035/2003 from the Comunidad de Madrid and RM03-002 from Instituto Nacional de Investigación Agraria y Tecnología Agraria y Alimentaria (INIA). We thank R. González for his advice and critical reading of the manuscript. The technical assistance of A. Fernández is greatly appreciated.

REFERENCES


