Mueller-Hinton Broth Undergoes Visible Oxidative Color Changes in the Presence of Peroxidase and Hydrogen Peroxide

LUCIANO GALEAZZI, GIUSEPPE GROPPA, AND SERGIO GIUNTA*
Sezione di Microbiologia, Laboratorio Clinico, Istituto di Ricovero e Cura a Carattere Scientifico I.N.R.C.A., 60100 Ancona, Italy

Received 29 November 1989/Accepted 19 June 1990

In the presence of peroxidase and hydrogen peroxide, Mueller-Hinton broth undergoes a slow but clearly detectable color change from pale yellow to dark yellow or brown. An investigation of this phenomenon led to the conclusion that it is the result of the oxidation of tyrosine, a major component of the broth. Indeed, tyrosine has long been known to oxidize upon treatment with peroxidase and hydrogen peroxide. The observations reported here, besides being curious for the clinical microbiologist, might deserve attention for the possible implications in the medium color darkening which sometimes happens during microbial growth.

Color changes of bacteriological media resulting from pigment production from certain bacteria is a common and well-known phenomenon. Sometimes these color changes are clearly evident, as in the case of pigment production by some Pseudomonas isolates; at other times, bacteriological broths and media reveal color shifts from pale yellow to dark yellow or brown. In this report, we present experimental data suggesting that apart from true pigment production from bacteria, medium components may contribute to the darkening of bacteriological media as a result of oxidative changes caused by enzymes and metabolites in the medium. In the course of investigations on oxygen toxicity in bacterial cells (9) utilizing exogenously added oxidants as well as oxidant scavengers, we observed that control tubes containing only bacteriological broth plus peroxidase (POD) and hydrogen peroxide underwent a slow but clearly visible oxidation resulting in the development of a dark yellow-brown color easily distinguishable from that of tubes containing broth alone, broth plus peroxidase, or broth plus hydrogen peroxide. Herein we describe the experimental data concerning these observations together with some preliminary results on the mechanism underlying the oxidative process.

The following bacteriological broth and broth components were used: Mueller-Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.); Mueller-Hinton broth (E. Merck AG, Darmstadt, Federal Republic of Germany), tryptone (pancreatic digest of casein) (Difco Laboratories, Detroit, Mich.), and casein acid hydrolysate (Sigma Chemical Co., St. Louis, Mo.). The enzymes and chemicals used were horseradish peroxidase, hydrogen peroxide (30% solution), tyrosine, and the other pure amino acids contained in casein; all were purchased from Sigma. All the spectrophotometric determinations reported were made by the Uvikon 860 spectrophotometer (Kontron, Milan, Italy). The experimental procedures consisted of the addition of a 10-μl portion of a horseradish peroxidase stock solution to 3 ml of bacteriological broth, to a final enzyme concentration of 1.5 IU. After the addition of hydrogen peroxide (final concentration, 0.005%), the color development of the broth incubated at room temperature was followed by monitoring the A440 in cuvettes with a 10-mm light path. The reported concentrations of POD and H2O2 gave an optimization of the reaction, which was, however, still clearly visually detected at very low concentrations of these reagents (POD, 0.15 U/ml; H2O2, 0.0015%). The wavelength of 400 nm was chosen after the examination of the absorption spectra of the reaction product(s). The same experimental procedure was performed with the components tryptone and casein acid hydrolysates, as well as with the amino acids constituents of casein, at the same concentration exactly reported by the manufacturer and reported in Table 1.

Thin-layer chromatography experiments on silica gel were performed by the procedure of Aeschbach et al. (1) in order to investigate the oxidation product formed. Thin-layer chromatography plastic sheet silica gels (60 F254; 20 by 20 mm; layer thickness, 0.2 mm; Merck) were used. A volume of 10 μl of the reaction mixture described in the experimental procedure and containing a final tyrosine concentration of 4 mM was loaded on the gel. The following solvent system was used: buten-1-ol-acetic acid-water (55:15:30, by volume). UV fluorescence was investigated by a Spectroline UV

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Casein pancreatic hydrolysate</th>
<th>Casein acid hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>2.440</td>
<td>0.940</td>
</tr>
<tr>
<td>Phe</td>
<td>3.200</td>
<td>1.140</td>
</tr>
<tr>
<td>Lys</td>
<td>5.560</td>
<td>2.440</td>
</tr>
<tr>
<td>His</td>
<td>1.680</td>
<td>0.660</td>
</tr>
<tr>
<td>Arg</td>
<td>3.760</td>
<td>0.690</td>
</tr>
<tr>
<td>Asp</td>
<td>6.860</td>
<td>3.660</td>
</tr>
<tr>
<td>Cys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>4.020</td>
<td>1.680</td>
</tr>
<tr>
<td>Ser</td>
<td>6.420</td>
<td>3.100</td>
</tr>
<tr>
<td>Pro</td>
<td>7.680</td>
<td>5.540</td>
</tr>
<tr>
<td>Glu</td>
<td>17.100</td>
<td>8.080</td>
</tr>
<tr>
<td>Gly</td>
<td>3.920</td>
<td>1.600</td>
</tr>
<tr>
<td>Ala</td>
<td>5.100</td>
<td>1.880</td>
</tr>
<tr>
<td>Val</td>
<td>6.680</td>
<td>2.460</td>
</tr>
<tr>
<td>Met</td>
<td>1.720</td>
<td>0.380</td>
</tr>
<tr>
<td>Ile</td>
<td>4.340</td>
<td>1.260</td>
</tr>
<tr>
<td>Leu</td>
<td>8.120</td>
<td>2.640</td>
</tr>
</tbody>
</table>

* Corresponding author.

* See reference 5.
transilluminator; colorimetric detections were performed by
the ninhydrin spray reagent, 0.1%, for chromatography
(Merck).

Figure 1 shows that the color change in a test tube
containing Mueller-Hinton broth plus peroxidase and hydro-
gen peroxide can be detected visually when compared with
control tubes containing medium alone, medium plus perox-
idase, or medium plus hydrogen peroxide. The color de-
velopment occurs slowly, and it can be seen visually after half
an hour. Figure 2 shows the absorption spectrum and peak
detection of the color developed in the test tube. The colored
component can be detected in the visible part of the spec-
trum as a shoulder at 400 to 480 nm (see the insert of Fig. 2).
Similar results were obtained with media from different
sources, including Mueller-Hinton broth (Becton-Dickinson)
and Mueller-Hinton broth (Merck). We have further ana-
lyzed the constituents in each broth in order to identify the
potential hydrogen donor(s) of the peroxidation observed.
Since casein hydrolysate was the major component of the
broths tested, we performed a similar oxidation analysis on
a sample of casein hydrolysate, a product consisting of
pancreatic hydrolysate of casein. Tryptone (Difco) was
tested at the same concentration present in Mueller-Hinton
broth, i.e., 17.5 mg/ml, and it produced a visible color
change and an absorption spectrum comparable to that of
Mueller-Hinton broth (data not reported). Analysis of casein
acid hydrolysate (Sigma) demonstrated slightly less darkening,
although it produced a similar absorption spectrum, with
a shoulder at 400 to 480 nm (data not shown). In order to
identify the casein component responsible for the color
development, we tested the individual amino acids known to
be the constituents of casein (5) and reported in Table 1.
When assayed under the same experimental conditions for
Mueller-Hinton and casein hydrolysate, color development
was obtained only with tyrosine, suggesting that this amino
acid was able to act as a weak hydrogen donor in the
peroxidase reaction. Indeed, working with tyrosine concen-
trations equal to those contained in the media tested (4 mM),
we detected the development of the same color and an
absorption spectrum exactly overlapping those of Mueller-
FIG. 4. Identification of dityrosine in the L-tyrosine sample (b) by thin-layer chromatography. a, L-Tyrosine control; b, sample of L-tyrosine oxidized with peroxidase and hydrogen peroxide. F, Fluorescent spot.

Hinton broth and casein hydrolysate, with a comparable shoulder at 400 to 480 nm in the visible region (see Fig. 3). The demonstration that tyrosine is the compound responsible for the peroxidase-induced color darkening also explained the previous finding that pancreatic casein hydrolysate showed a higher absorbance than acid casein hydrolysate in the same experimental conditions, since the pancreatic hydrolysate contains more tyrosine than the acid hydrolysate (Table 1).

The nature of the oxidative modification of tyrosine upon treatment with peroxidase and hydrogen peroxide was revealed by the thin-layer chromatography experiments. Figure 4 shows that upon addition of peroxidase and hydrogen to tyrosine, a novel ninhydrin-positive spot can be detected. This slowly migrating component fluoresces under UV light, suggesting that dityrosine has been formed (1).

Color changes in bacteriological cultures are often the result of bacterial pigment production; however, darkening may also result from oxidation of bacterial metabolites. Herein we have shown that oxidation of the normal constituents of bacteriological media are also potential sources of color changes. Peroxidases are enzymes with a narrow specificity of hydrogen acceptors. However, the specificity of these enzymes for the hydrogen donor is low, and a number of compounds are active (3). The donor may be an amine or a phenol. Consequently, the list of potential donor compounds is wide (8, 10). The finding that tyrosine can act as a substrate in a peroxidase reaction is not surprising; Gross and Sizer (11) first described the formation of dityrosine (3,3′-bityrosine), which they obtained by oxidation of L-tyrosine with peroxidase and hydrogen peroxide. They demonstrated that in certain conditions, trityrosine also can be formed (11). The mechanism of the reaction can be formulated as phenolic coupling of two phenoxyl radicals of tyrosine (1, 2, 11). Therefore, in an in vitro system containing 4 mM tyrosine together with peroxidase and hydrogen peroxide, this amino acid can react in a peroxidase reaction, resulting in the oxidative darkening of bacteriological media. This finding may be of potential significance, since bacterial peroxidases can be either constitutive or inducible enzymes and hydrogen peroxide production may result from bacterial metabolism. The inhibition of the growth of one bacterial species by the hydrogen peroxide generated by another species is a well-recognized mechanism of bacterial antagonism (4, 6) and has clinical significance (6). Therefore, an important implication of our findings is that tyrosine, a weak yet efficient hydrogen donor, upon production of hydrogen peroxide by some bacterial cells, may act as a cosubstrate for the bacterial peroxidases in a reaction similar to that here reported, thereby activating a scavenging pathway for this cytotoxic growth-limiting compound (hydrogen peroxide).

LITERATURE CITED