Polyacrylamide Gel Electrophoresis of Isoenzymes from *Entamoeba* Species

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In this preliminary report, we describe a polyacrylamide gel electrophoresis technique for the resolution of isoenzyme patterns of four isolates of *Entamoeba histolytica* and one isolate of *Entamoeba coli*. Our findings were similar to previous findings for three enzyme systems: maleic enzyme (malate dehydrogenase [EC 1.1.1.40]), hexokinase (EC 2.7.1.1), and phosphoglucomutase (EC 2.7.5.1). We found preliminary evidence that glucosephosphate isomerase (EC 5.3.1.9) may also differentiate invasive amoebae from noninvasive amoebae, when the isoenzymes are separated by polyacrylamide gel electrophoresis, whereas this differentiation is not evident with starch-gel electrophoresis. We used an R_f system to relate isoenzyme band mobility to the migration distance of a standard *E. histolytica* strain (HK-9). The numerical identification of isoenzyme bands can simplify the grouping of isolates into zymodemes.

Sargeaunt and Williams (6, 7) and Sargeaunt and co-workers (8–10) have used starch-gel electrophoresis of selected isoenzymes as a basis for distinguishing pathogenic (invasive) isolates from nonpathogenic isolates of *Entamoeba histolytica*. This method also provides an additional means for identifying and separating various genera of amoebae inhabiting the human intestine.

The following report is an initial evaluation of polyacrylamide as the supporting matrix for electrophoresis of enzymes from extracts of *E. histolytica* and *Entamoeba coli* isolates. This method yielded results similar to those previously reported (6–10). It further confirmed the value of isoenzyme patterns as a key to taxonomy and as a means of typing other biological characteristics of the intestinal amoebae. A detailed description of isoenzyme electrophoresis and enzyme detection is given by Harris and Hopkinson (5).

MATERIALS AND METHODS

All chemicals and reagents were reagent grade or better. Tris was obtained from Schwarz/Mann, Spring Valley, N.Y. Acrylamide gel reagents were obtained from Bio-Rad Laboratories, Richmond, Calif., and reagents for enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo.

Four isolates of *E. histolytica* and one of *E. coli* (Table 1) were examined for isoenzyme patterns. All were grown in TYGM medium (2) in the presence of mixed bacterial flora, except for one (HK-9) which was grown axenically in Diamond TPS-1 medium (1). Organisms were recovered from cultures by first chill-

ing tubes on wet ice for 10 to 20 min to detach amoebae adhering to the glass. The amoebae were sedimented by centrifuging at 350 × *g* for 15 min at room temperature. The supernatant containing most of the bacteria was recovered and centrifuged at 30,000 × *g* for 15 min. The two pellets (amoebae and bacteria) were each taken up in a volume equal to that of the amoebic sediment in deionized water containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM 6-aminocaproic acid (5) to stabilize target enzymes and inhibit proteases. Tubes containing the amoebic and bacterial pellets were frozen in a mechanical freezer at −50°C. On the next day, the pellets were thawed in a 37°C water bath, refrozen in a dry ice-methanol slurry, and again thawed at 37°C. After one additional cycle of freezing and thawing, the enzyme extracts were centrifuged at 30,000 × *g* for 30 min at 4°C. The soluble supernatants were divided into small volumes (10 to 25 μl) and stored at −50°C.

Polyacrylamide gel electrophoresis was carried out in a horizontal gel (240 by 120 by 2 mm; Multiphore 2117 system; LKB Instruments, Inc., Gaithersburg, Md.). Homogeneous gels containing 5% acrylamide (5.1% T, 2.6% C) were prepared with 0.015 M Tris-NaOH–0.015 M maleic acid–3.15 mM $MgCl_2$–1.5 mM EDTA (pH 7.4) buffer. The gels were allowed to stand overnight at room temperature before use. Gel slabs with the supporting plate were placed on a cooling platform (10°C) and connected to the electrode buffer (0.1 M Tris-NaOH, 0.1 M maleic acid, 0.021 M $MgCl_2$, 0.01 M EDTA [pH 7.4]) with Ultra Wicks (Bio-Rad Laboratories). The gels were preelectrophoresed at 50 mA for 40 min. Enzyme samples (2- to 10-μl volumes) were added and initially electrophoresed into the gels at 60 V for 5 min and then at 175 V for 2 h. Upon completion of electrophoresis, each gel was removed from its glass support, transferred to a glass dish, and immersed in the appropriate staining solution (Table

TABLE 1. Stock amoeba isolates

Isolate	Species	Type of culture	Host status	Reference
HK-9	*E. histolytica*	Axenic	Acute amebiasis	4
HSC-1	*E. histolytica*	MBF[a]	Cyst passer; sibling of patient with amoebic liver abscess; isolate invasive in guinea pigs	(G. R. Healy, G. S. Visvesvara, and W. Lushbaugh, unpublished data)
Webb	*E. histolytica*	MBF	Cyst passer; isolate noninvasive in guinea pigs	
CDC-0382-1	*E. histolytica*	MBF	Cyst passer	
CDC-2	*E. coli*	MBF	Cyst passer	

[a] MBF, Mixed bacterial flora.

2). The dishes were covered and incubated for 45 to 60 min at 37°C. The staining solution was removed; the gels were rinsed with deionized water and finally fixed with 7% (vol/vol) acetic acid. Stained gels were photographed, and the gels were discarded.

Extracts from *E. histolytica* (HK-9) were included in all electrophoretic runs. The migration distance of the enzyme band(s), measured from the leading edge of the sample slot to the center of the stained enzyme, was recorded. An R_f value was calculated for each isoenzyme, based on the migration distance of the HK-9 band ($R_f = 1.0$) located closest to the sample application point.

RESULTS

Isoenzyme patterns from the amoebae and the bacterial controls are shown in Fig. 1, 2, 3, and 4. Because bacteria and amoebae could not be cleanly separated, there was frequently some cross-contamination between the two preparations. Generally, bacterial enzymes migrate farther than enzymes derived from amoebae (6). Occasionally, a faint band of activity in the bacterial control occurred at the same relative mobility as a strong band in the amoebic preparation. In such cases, the faint band was assumed to be of amoebic origin. Similary, faint bands in both the amoebic preparation and the bacterial controls may have been due to phagocytosed bacteria; thus, such bands (Fig. 3, lanes 4 and 5) were considered to be of bacterial origin.

Malic enzyme (L-malate:NADP$^+$ oxidoreductase [oxaloacetate-decarboxylating] did not appear in the bacterial control samples (Fig. 1). All

TABLE 2. Composition of enzyme-indicating systems[a]

Enzyme	pH of buffer[b]	Amt of coenzyme (NADP [mg])	Substrate	Amt of linking enzyme (glucose-6-phosphate dehydrogenase [U])	MgCl$_2$ concn (mM)	Amt (mg) of:		
						MTT tetrazolium[c]	PMS[d]	ATP
Maleic enzyme	7.4	20	3 mM maleic acid (pH 7.0) with NaOH (0.3 ml of 0.1 M stock)		10 (10 ml of 0.1 M stock)	20	4	
Hexokinase	7.4	24	Glucose (190 mg)	96	7.6 (7.6 ml of 0.1 M stock)	24	4	72
Phosphoglucomutase	7.4	10	Sodium-glucose-1-phosphate (200 mg)	40	10 (10 ml of 0.1 M stock)	20	4	
Glucosephosphate isomerase	8.0	10	Fructose-6-phosphate (36 mg)	20	10 (10 ml of 0.1 M stock)	10	4	

[a] Ingredients for 100 ml of staining solution. Stock solutions were mixed, the remaining ingredients were added and dissolved, and the volume was adjusted to 100 ml with deionized water. This procedure is a modification of that of Sargeaunt et al. (10).
[b] Buffer is 0.1 M Tris-hydrochloride (10 ml of 1.0 M stock).
[c] MTT tetrazolium, Methyl thiazolyl tetrazolium.
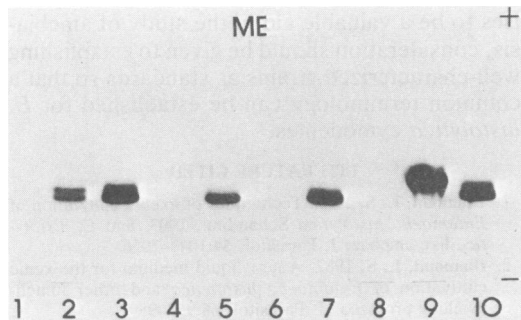[d] PMS, Phenazine methosulfate.

FIG. 1. Typical zymogram stained for maleic enzyme (ME). Lanes: 1, bacterial control for 2; 2, Webb; 3, HK-9, axenic; 4, bacterial control for 5; 5, HSC-1; 6, bacterial control for 7; 7, CDC-0382-1; 8, bacterial control for 9; 9, *E. coli* CDC-2; 10, HK-9, axenic.
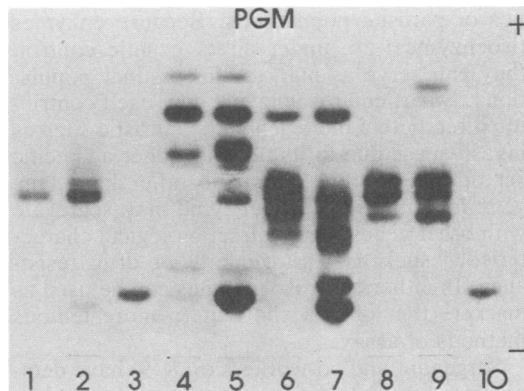

FIG. 3. Typical zymogram stained for phosphoglucomutase (PGM). Lanes are as described in the legend to Fig. 1.

of the *E. histolytica* isolates tested contained only a single isoenzyme moiety ($R_f = 1.0$). Some isolates (Fig. 1, lanes 2, 3, and 10) may have had two poorly separated bands but were considered to have single bands. The *E. coli* isolate contained an isoenzyme with a faster mobility ($R_f = 1.2$), thus differentiating this species. This observation agrees well with that of Sargeaunt and Williams (6).

Two distinctive patterns of hexokinase activity were detected (Fig. 2). Noninvasive isolates of *E. histolytica* (Webb, lane 2, and CDC-0382-1, lane 7) exhibited a two-band pattern with R_f values of 0.7 and 1.1, respectively. The invasive isolates (HK-9 and HSC-1) also had a two-band pattern, but with R_f values of 1.0 and 1.4, respectively. Hexokinase activity was not detected in the *E. coli* isolate.

Phosphoglucomutase activity appeared to differentiate invasive from noninvasive isolates of *E. histolytica*, as well as this species from *E. coli* (Fig. 3). The invasive isolates HK-9 and HSC-1 had a single band ($R_f = 1.0$), whereas the two noninvasive isolates had unique patterns. The

Webb isolate had a single band ($R_f = 0.8$), and CDC-0382-1 had two bands ($R_f = 0.8$ and 1.2). The *E. coli* isolate had a single fast-moving band ($R_f = 3.1$).

Glucosephosphate isomerase activity also appeared to differentiate invasive from noninvasive isolates (Fig. 4). Both invasive isolates demonstrated a single enzyme band ($R_f = 1.0$). The isolate designated Webb had a single band ($R_f = 0.9$), and CDC-0382-1 had two bands ($R_f = 0.9$ and 1.3). The single band of glucosephosphate isomerase activity found in the *E. coli* isolate had an R_f value of 4.1. Our findings differ from those of Sargeaunt and Williams (10), who found one isoenzyme band which was common to all *E. histolytica* isolates, with some isolates containing an additional band(s).

DISCUSSION

Isoenzyme markers are of great potential value for evaluating strain (and isolate) characteris-
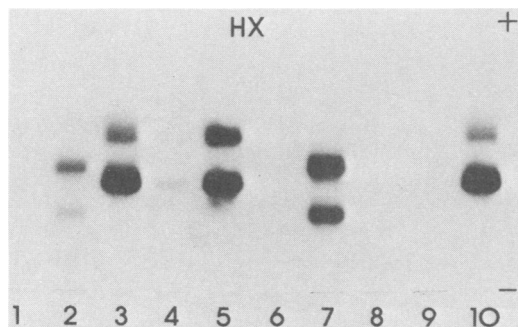

FIG. 2. Typical zymogram stained for hexokinase (HX). Lanes are as described in the legend to Fig. 1.
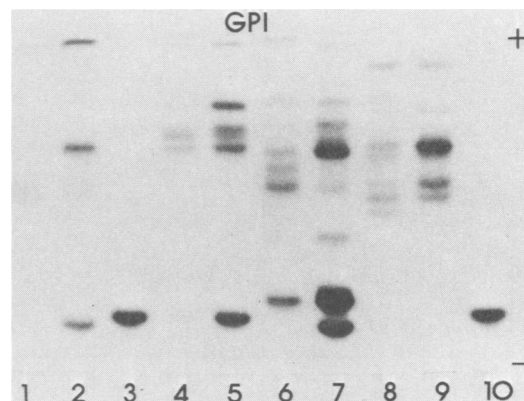

FIG. 4. Typical zymogram stained for glucosephosphate isomerase (GPI). Lanes are as described in the legend to Fig. 1.

tics of parasite populations. Because enzymes (isoenzymes) are under direct genetic control, they can serve as markers for distinct populations. A particular isoenzyme may itself contribute directly to a biological characteristic such as invasiveness due to its activity under a specific set of circumstances. On the other hand, the gene for a particular isoenzyme may segregate with other genes which direct biological characteristics such as pathogenicity or drug resistance. In either case, isoenzymes can be used as markers that offer a shortcut to more tedious methods of assay.

Sargeaunt and co-workers (6, 8, 9) have demonstrated the value of starch-gel isoenzyme electrophoresis in typing intestinal amoebae for speciation and for invasiveness. We have evaluated a PAGE-isoenzyme system and have found it to yield results similar to the findings with the starch-gel system. We believe that the PAGE system may offer future advantages, in that it can be prepared in a gradient configuration (3) which aids in separating protein molecules on the basis of size and shape as well as differences in charge.

Although we report here the characterization of only a few isolates, examination of additional *E. histolytica* isolates by our methods has yielded results which agree with these data. We have not yet grouped *E. histolytica* isolates into zymodemes as have workers in England (8), but we expect to as more isolates are examined.

Our studies are continuing as new isolates are obtained. Additional enzyme systems will be evaluated as part of an ongoing program. Because this endeavor is in its infancy and promises to be a valuable aid in the study of amebiasis, consideration should be given to establishing well-characterized strains as standards so that a common terminology can be established for *E. histolytica* zymodemes.

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