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Naphthylamidase Activity of *Leptospira*[1]

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Extracts of 18 serotypes of the genus *Leptospira* were found to possess naphthylamidase activity, and differences in the pathogenic and saprophytic strains were noted. The former exhibited a preference for the leucyl naphthylamide substrate, whereas the latter demonstrated greater hydrolysis of alanyl naphthylamide. With the leucyl naphthylamide as substrate, pathogenic strains showed 10 to 20 times higher naphthylamidase activity than saprophytic strains. Optimal temperature and *p*H for enzymatic hydrolysis also differed between pathogenic and saprophytic strains. Maximal enzymatic activities for pathogenic and saprophytic naphthylamidases were 41 and 37 C, respectively, at *p*H 8.0 to 8.5. The *p*H and temperature optima suggested that the leptospiral enzyme activity was not leucine aminopeptidase.

Many criteria have been used in attempts to distinguish the pathogenic from the nonpathogenic leptospirae in an attempt to elucidate the mechanism of pathogenesis. Some of the more recent identifying characteristics have been the resistance to copper sulfate (2), egg yolk decomposition (3), susceptibility to 8-azaguanine (7), and range of growth temperatures (6).

In a study of leptospiral enzyme patterns determined by starch-gel electrophoresis, Green, Goldberg, and Blenden (5) found that pathogenic strains possessed naphthylamidase activity, whereas the nonpathogens tested were devoid of such activity. This paper is an extension of those observations.

MATERIALS AND METHODS

Leptospira serotypes were cultured, and intracellular enzymes were extracted as described previously (5). The method of Goldbarg and Rutenberg (4) was employed to determine naphthylamidase activity for each of the strains used, since this technique is more sensitive than the electrophoretic procedure used in the earlier work.

A 1-ml amount of the cell-free extract and 1 ml of L-leucyl-β-naphthylamide hydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio), 2.74 mM in 0.1 M phosphate buffer (*p*H 8.0), were incubated at 37 C for 2 hr, whereupon enzymatic hydrolysis was terminated by the addition of 1 ml of 40% trichloroacetic acid. The mixture was then centrifuged at 1,640 × *g* for 15 min to sediment the precipitated protein. A 1-ml amount of the clear supernatant fluid was removed and diazotization of the β-naphthylamine released was carried out with 1 ml of 0.1% sodium nitrite (Mallinckrodt Chemical Works, St. Louis, Mo.). After 5 min, excess sodium nitrite was decomposed by the addition of 1 ml of 0.5% ammonium sulfamate (Mann Fine Chemicals, Inc., New York, N.Y.). A 2-ml amount of *N*-(1-naphthyl)ethylene diamine dihydrochloride solution (Mann Fine Chemicals) was added to each tube 5 min later to yield a blue azo dye. After 30 min, when the color reached maximum intensity, the absorption was determined at 580 nm on a Coleman Junior spectophotometer. This color density was converted to micrograms of β-naphthylamine liberated by means of a standard curve employing commercial β-naphthylamine (Mann Fine Chemicals).

The amount of protein in each 1-ml sample of extract was determined by the method of Lowry et al. (8). Enzymatic activity was expressed as micrograms of β-naphthylamine liberated per hour per milligram of protein. Appropriate controls substituting distilled water for the crude enzyme extract were employed in each case. Extracts were stored at −67 C and assayed within 1 week after preparation.

Leucine aminopeptidase, a leucyl-β-naphthylamide-hydrolyzing enzyme from hog kidney (Nutritional Biochemicals Corp.), was used to provide a comparison with the crude leptospiral enzyme preparations. Five micrograms of this enzyme was allowed to react with the substrate under the same conditions as above. In all experiments with leucine aminopeptidase, 6 mM magnesium ion was required for enzymatic activity.

Optimal temperature and *p*H range of enzymatic activity for leucine aminopeptidase and the leptospiral enzymes were also determined by the above methods.

Use of additional substrate. DL-Alanyl-β-naphthylamide (Nutritional Biochemicals Corp.) was employed in the same manner as the leucyl-β-naphthylamide to

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ascertain the substrate specificity of both the leptospiral extracts and the commercial leucine aminopeptidase, but the assays were carried out at *p*H 7.0, the reported *p*H optimum for hydrolysis of the alanyl substrate (9).

TABLE 1. *Naphthylamidase activity of leptospiral serotypes*

Serotype	Strain	Activity[a]
L. andamana		36.7
L. atlantae		360.0
L. australis	Ballico	402.0
L. australis	Akiyama A	291.0
L. autumnalis	Rachmat	219.0
L. ballum	s-102	204.0
L. bataviae	Van Tienen	324.0
L. biflexa	CDC	2.4
L. biflexa	Sao Paulo	16.9
L. canicola	Hond Utrecht	27.3
L. canicola	Moulton	264.0
L. hebdomadis	Hebdomadis	243.0
L. hyos	LT-81	180.0
L. icterohaemorrhagiae	CF-1	387.0
L. icterohaemorrhagiae	LT-351	152.0
L. pomona	4822	202.0
L. pyrogenes	Salinem	342.0
L. sejroe	Mallersdorf	197.0

[a] Naphthylamidase activity is expressed as micrograms of β-naphthylamine liberated per hour per milligram of protein with L-leucyl-β-naphthylamide hydrochloride as the substrate.

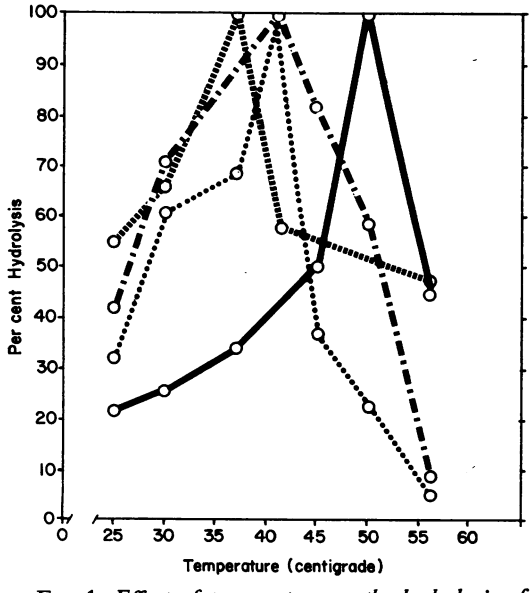

FIG. 1. *Effect of temperature on the hydrolysis of L-leucyl-β-naphthylamide. Symbols: solid line, leucine aminopeptidase; dotted line, L. atlantae; alternate long and short dashes, L. pomona 4822; dashed line, L. biflexa Sao Paulo.*

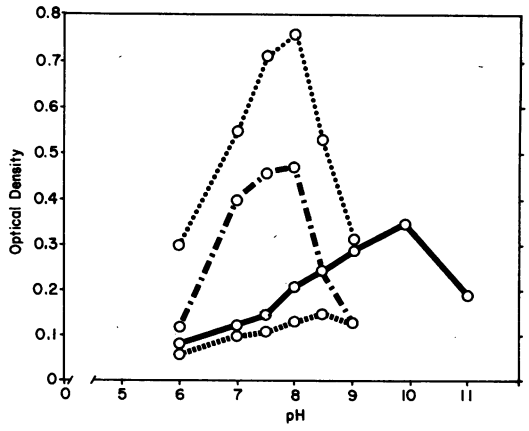

FIG. 2. *Effect of pH on the hydrolysis of L-leucyl-β-naphthylamide. Symbols: solid line, leucine aminopeptidase; dotted line, L. atlantae; alternate long and short dashes, L. ballum M 127; dashed line, L. andamana.*

TABLE 2. *Comparative hydrolysis of DL-alanyl- and L-leucyl-β-naphthylamide by strains of Leptospira*

Serotype	Strain	DL-Alanyl substrate[a]	L-Leucyl substrate[a]
L. andamana		370.0	36.7
L. atlantae		188.0	360.0
L. biflexa	Sao Paulo	152.0	16.9
L. canicola	Hond Utrecht	103.0	27.3
L. icterohaemorrhagiae	CF-1	171.0	387.0
L. pomona	4822	95.0	220.0

[a] Enzymatic activity is expressed as micrograms of β-naphthylamine liberated per hour per milligram of protein with the specified substrate.

RESULTS AND DISCUSSION

The colorimetric method of Goldbarg and Rutenberg (4) employed to determine hydrolysis of L-leucyl-β-naphthylamide proved to be more sensitive than the starch-gel electrophoretic technique of Green et al. (5). All leptospiral strains tested were found to possess some leucyl naphthylamide-hydrolyzing ability. The relative activities of the strains tested are listed in Table 1, in which it can be seen that more than 100-fold variation in activity exists among certain saprophytic and pathogenic strains of *Leptospira*. The nonpathogenic biflexa serotypes exhibited the least leucyl-hydrolyzing ability.

L. andamana, which previous investigators (2, 3, 11) showed to be related to the saprophytic biflexa strains, demonstrated a low activity which

was similar to the nonpathogens. This indicates once more that *L. andamana* is more closely related to the biflexa group and should be classified as a nonpathogen.

Three representive serotypes were selected for determination of the effect of temperature on the activity of their respective naphthylamidases. The optimal enzymatic activity of the leptospiral enzyme was at 37 C for saprophytes and at 41 C for pathogens. In contrast, leucine aminopeptidase showed its optimal hydrolytic activity at 50 C (Fig. 1).

The optimal *p*H for enzymatic hydrolysis of the leucyl-β-naphthylamide was found to range between 7.5 and 8.5 for the leptospiral strains tested. No clear-cut difference was noted between pathogens and nonpathogens (Fig. 2).

Pathogenic leptospiral enzymes and leucine aminopeptidase exhibited a preferential cleavage of the leucyl over the alanyl naphthylamide. In contrast, *L. biflexa* showed a definite preference for the alanyl substrate. This strain hydrolyzed the alanyl naphthylamide 2 to 10 times as effectively as the leucyl substrate (Table 2).

The differences in amino acid naphthylamide preference among the pathogenic and saprophytic enzymes would seem to indicate that at least two different enzymes (or isozymes) are involved in this hydrolysis. The divergence in optimal temperature of pathogenic and saprophytic enzymes supports this conclusion. Previous researchers have postulated the existence of more than one naphthylamidase (1, 10).

The leptospiral enzymes and leucine aminopeptidase differed markedly in optimal temperature and *p*H values for hydrolysis of the leucyl-β-naphthylamide, indicating that the leptospiral enzyme responsible for hydrolyzing amino acid naphthylamides is not leucine aminopeptidase. The enzyme or enzymes in *Leptospira* are most likely true naphthylamidases. The modifying influence of the amino acid side chain is evident from the different enzymatic activities with the alanyl and leucyl naphthylamide substrates. It would be valuable to use a greater variety of amino acid naphthylamides to determine the hydrolysis patterns for the various serotypes of *Leptospira*.

No additional metal ion was required for enzymatic activity of the leptospiral enzyme. Leucine aminopetidase and some of the reported naphthylamidas have been found to require such ions as zinc, cobalt, manganese, and magnesium.

The in vivo role of naphthylamidase remains obscure and its role in pathogenicity cannot yet be determined. However, it should be noted that investigators have used elevation of serum naphthylamidase levels as an indication of cancer and other diseases of the liver or bile ducts (4). Often in the more severe manifestations of leptospirosis liver involvement is seen. It is therefore possible that naphthylamidase plays a role in the virulence of the *Leptospira*.

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