Aminopeptidase Activity of *Leptospira* Strains

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A total of 15 cultures of *Leptospira* were examined for aminopeptidase activity using 22 aminoacyl- β -naphthylamide substrates. Activity was demonstrated in each of the cultures. Extracts from serovars of *Leptospira interrogans* preferentially hydrolysed the same range of substrates. The level of hydrolysis of the preferred substrates for the seven strains of *L. interrogans* was distinctively higher than that demonstrated for the six *Leptospira biflexa* strains. Extracts from cultures of *Leptospira illini* and *Leptospira parva* sp. nov. exhibited profiles different to those demonstrated for the other 13 leptospiral cultures examined.

INTRODUCTION

The leptospire is unusual amongst the aerobic prokaryotes, as little or no use has been made of biochemical characters in their taxonomy or identification. The genus *Leptospira* has traditionally been divided into two groups or complexes designated 'interrogans' and 'biflexa' (Turner, 1974). The complexes have now attained official acceptance as the distinct and genuine species *Leptospira interrogans* and *Leptospira biflexa* (Skerman *et al.*, 1980), and contain the parasitic and saprophytic strains, respectively. Classification and subsequent identification of *Leptospira* is primarily based on serological analysis of cellular antigens and has resulted in subdivision into many serovars which, for practical purposes, are arranged into major serogroups of closely related serovars. Phenotypic characteristics have been examined to a limited degree and although of little taxonomic value they appear useful for distinguishing between pathogenic and saprophytic strains (Stalheim, 1973).

The possible use of enzymes in the classification of *Leptospira* was suggested following a study of a range of enzymes by electrophoresis (Green *et al.*, 1967). The recognition of a naphthylamidase amongst these prompted further examination of leptospire for specific naphthylamidase activity (Burton *et al.*, 1970). Aminopeptidases have been demonstrated in a variety of micro-organisms (Watson, 1976), and differences in substrate specificity have been used to distinguish between species of bacteria (Watson & Perrine, 1975), fungi (Lee *et al.*, 1975) and mycoplasmas (Neill & Ball, 1980).

In this study a number of leptospiral serovars were examined for aminopeptidase activity using 22 aminoacyl- β -naphthylamide substrates and compared their substrate specificities.

METHODS

Organisms. Details of the 15 *Leptospira* strains employed in this study are presented in Table 1. The cultures were maintained by weekly subculture in polysorbate-bovine serum albumin medium (Johnson & Harris, 1967) containing 1% (v/v) rabbit serum and 0.15% (v/v) purified agar (Baltimore Biological Laboratories). Rabbit serum used in the medium was a pool of sera from more than 10 rabbits serologically negative for *Leptospira*. The serum was inactivated at 56 °C for 30 min and stored at –20 °C until required. The complete medium was stored at 4 °C.

Preparation of cell extracts. Each strain was inoculated into 100 ml volumes of liquid medium with or without serum. After incubation at 30 °C for 7 d organisms were harvested by centrifugation at 10000 g for 1 h. The resulting supernatants were used as a source of crude extracellular enzyme. The deposit was washed in 5–10 ml of 0.1 M-Tris/HCl buffer pH 8.0, centrifuged as above and the washing was added to the original supernatant which was held at –20 °C until required. The final cell deposit was resuspended in 10 ml 0.1 M-Tris/HCl buffer. Cell suspensions were ultrasonicated three times for periods of 45 s at 30 s intervals at maximum amplitude (12 μ m), using a disintegrator (MSE mk II). During sonication the sample container was packed in ice. The sonicate was

Table 1. *Organisms used*

Species	Serogroup	Serovar	Strain*
<i>L. interrogans</i>	Hebdomadis	Hardjo	Hardjoprajitno
	Hebdomadis	Mini	Sari
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	Canicola	Canicola	Hond Utrecht IV
	Grippotyphosa	Grippotyphosa	CHI
	Pomona	Pomona	Pomona
	Australis	Lora	Lora
<i>L. biflexa</i>	Semarang	Semarang	Veldrat S173
	Semarang	Patoc	Patoc 1
	Andamanda	Andamanda	CH11
	—	—	762
	—	—	691
	—	—	796
		Illini	3055
<i>L. parva</i> sp. nov.†	—	—	—

* As obtained from the Leptospiral Reference Laboratory, Colindale, London, U.K.

† Hovind-Hougen *et al.* (1981).

centrifuged for 1 h at 20000 g. The resulting supernatant was assayed for enzyme activity, which was regarded as intracellular in origin. The extracts were stored at -20°C . Total protein in the extract was determined by the method of Hartree (1972).

Chemicals. The β -naphthylamide derivatives of alanine, isoleucine, serine, cystine and leucine (the latter as the hydrochloride) were supplied by Sigma; those of valine, phenylalanine, tyrosine, tryptophan, arginine, lysine (as carbonate), glycine and proline (as hydrobromides) and pyrrolidonyl- β -naphthylamide were obtained from I.C.N. Pharmaceuticals, Cleveland, Ohio, U.S.A.; the derivatives of histidine, 4-methoxyleucine, 4-hydroxyproline, 1-aspartyl- β -naphthylamide and 5-glutamyl- β -naphthylamide were from Koch-Light; the derivatives of ornithine and glutamine were obtained from Cambridge Research Biochemicals, U.K. The β -naphthylamides were all derivatives of L-amino acids.

Enzyme assay. Aminopeptidase activity was measured by following the hydrolysis of aminoacyl- β -naphthylamides. Release of fluorescent β -naphthylamine was measured using a Perkin-Elmer spectrophotometer, model 204A (excitation λ 337 nm; emission λ 408 nm). Substrates (10^{-4} M) were dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, and then dispensed in 2 ml amounts into glass Bijou bottles, capped and sterilized by autoclaving for 10 min at 121°C . L-Glutamyl- β -naphthylamide, which hydrolyses on autoclaving, was filter-sterilized prior to dispensing. Sterilized substrate solutions were stored at 4°C until required. The extract under test was added (0.2 ml) to each of the buffered substrate solutions (2 ml) in duplicate, the latter previously warmed to 37°C . Incubation was continued for 4 h, after which the mean fluorescence of duplicate reaction mixtures was determined. Substrate instability was corrected for by subtracting the mean fluorescence for the appropriate uninoculated buffered substrate solutions. The arbitrary fluorescence units were converted to μmol β -naphthylamine released, using a linear calibration curve prepared with 5–100 μmol β -naphthylamine. Aminopeptidase activity of cell extracts was recorded as μmol β -naphthylamine formed (mg protein) $^{-1}$.

RESULTS AND DISCUSSION

Aminopeptidase activity demonstrated using the crude bacterial extracellular preparations was minimal, as little or no increase in hydrolysis was observed relative to that obtained with the uninoculated culture medium. In preliminary experiments using uninoculated medium prepared without serum, the maximum hydrolysis demonstrated with any substrate was $<1.5 \mu\text{mol}$ β -naphthylamine released (mg protein) $^{-1}$. Uninoculated medium containing serum, hydrolysed a larger range of substrates, due to serum aminopeptidase; however, the activity remained relatively low with all substrates, ranging from 0 to $14 \mu\text{mol}$ β -naphthylamine released (mg protein) $^{-1}$.

Some hydrolytic activity was demonstrated with uninoculated medium treated similarly to the preparations of the intracellular extracts. This activity was again relatively low compared to the microbial intracellular activity and was only marginally affected by the incorporation of serum in the medium. Using extracts prepared from medium with and without serum, the hydrolysis observed ranged from 0 to $<22 \mu\text{mol}$ β -naphthylamine released (mg protein) $^{-1}$. It was therefore considered that the hydrolytic activity of the medium constituents, extracted during the enzyme

Table 2. Comparison of aminopeptidase activity of extracts of *L. interrogans* serovars grown in medium prepared with and without serum

Extracts were prepared from cultures grown in medium with and without 1% (v/v) rabbit serum (see Methods). Enzyme activities were measured after 4 h incubation. Duplicate assays were carried out on two samples from each culture. The results are the mean activities of extracts from three individual cultures of each strain; the s.e.s are shown.

Substrate*	Aminopeptidase activity [$\mu\text{mol } \beta\text{-naphthylamine released in 4 h (mg protein)}^{-1}$]			
	<i>L. pomona</i>		<i>L. hardjo</i>	
	Grown without serum	Grown with serum	Grown without serum	Grown with serum
Ala-ONA	203.1 $\pm$ 79.9	275.0 $\pm$ 30.8	135.9 $\pm$ 38.7	168.1 $\pm$ 29.9
Val-ONA	22.4 $\pm$ 7.5	31.0 $\pm$ 8.1	2.2 $\pm$ 0.7	36.0 $\pm$ 9.2
Leu-ONA	246.8 $\pm$ 61.7	321.0 $\pm$ 10.3	164.9 $\pm$ 30.3	232.1 $\pm$ 62.7
Ile-ONA	26.8 $\pm$ 6.1	46.9 $\pm$ 5.8	4.1 $\pm$ 1.2	40.1 $\pm$ 8.4
Phe-ONA	68.8 $\pm$ 11.2	287.7 $\pm$ 16.4	75.4 $\pm$ 21.1	86.7 $\pm$ 22.5
(4-OMe)Leu-ONA	149.2 $\pm$ 38.2	186.3 $\pm$ 12.4	88.3 $\pm$ 18.6	138.6 $\pm$ 33.2
Asp ¹ -ONA	441.2 $\pm$ 89.0	358.3 $\pm$ 20.4	142.6 $\pm$ 54.4	383.1 $\pm$ 48.0
Glu ² -ONA	31.0 $\pm$ 9.6	61.2 $\pm$ 3.9	13.0 $\pm$ 2.1	61.6 $\pm$ 7.6
Lys-ONA	341.4 $\pm$ 103.8	358.2 $\pm$ 58.5	254.1 $\pm$ 43.3	399.1 $\pm$ 38.3
Arg-ONA	223.3 $\pm$ 24.5	489.3 $\pm$ 18.0	236.5 $\pm$ 46.4	140.4 $\pm$ 49.6
Ser-ONA	49.1 $\pm$ 6.7	99.1 $\pm$ 11.3	16.9 $\pm$ 4.0	56.4 $\pm$ 11.2
Tyr-ONA	78.9 $\pm$ 22.9	290.3 $\pm$ 19.7	62.1 $\pm$ 18.1	98.6 $\pm$ 27.7
Trp-ONA	38.1 $\pm$ 10.9	93.5 $\pm$ 5.7	17.3 $\pm$ 4.6	70.6 $\pm$ 14.5
Gly-ONA	39.2 $\pm$ 7.9	80.0 $\pm$ 13.6	26.6 $\pm$ 12.5	43.8 $\pm$ 3.0
Cys-ONA	44.0 $\pm$ 5.6	66.8 $\pm$ 7.1	31.4 $\pm$ 6.3	43.9 $\pm$ 18.1
Met-ONA	80.9 $\pm$ 26.5	157.3 $\pm$ 9.2	56.6 $\pm$ 14.7	78.0 $\pm$ 8.2
His-ONA	37.6 $\pm$ 8.0	139.2 $\pm$ 2.9	29.7 $\pm$ 8.8	73.6 $\pm$ 7.1
Pro-ONA	23.7 $\pm$ 2.3	41.7 $\pm$ 1.4	3.6 $\pm$ 1.4	29.7 $\pm$ 4.7
(4-OH)Pro-ONA	9.6 $\pm$ 1.9	17.5 $\pm$ 1.6	0.4 $\pm$ 0.4	3.9 $\pm$ 1.5
Pyr-ONA†	6.0 $\pm$ 1.7	17.7 $\pm$ 0.9	1.5 $\pm$ 0.7	10.3 $\pm$ 4.0
Orn-ONA	288.9 $\pm$ 77.7	308.2 $\pm$ 12.5	179.1 $\pm$ 20.7	107.7 $\pm$ 19.2
Gln-ONA	475.1 $\pm$ 177.7	331.7 $\pm$ 14.3	227.9 $\pm$ 29.3	375.7 $\pm$ 55.4

* -ONA, β -Naphthylamine derivative.

† Pyr-ONA, Pyrrolidonyl- β -naphthylamide.

preparation, contributed little to the activity of the leptospiral extracts. This was particularly evident for strains of *L. interrogans*. Although the contribution of serum aminopeptidase(s) to the activity of the leptospiral extracts was regarded as minimal, the latter activity was influenced by incorporation of serum into the medium. In an attempt to exclude this serum variable, eventual comparison of the strains under test was made using extracts of cultures grown in serum-free medium.

Intracellular aminopeptidase activity was demonstrated in each of the cultures examined. This hydrolytic activity was in general increased when rabbit serum was incorporated into the culture medium as illustrated by the two examples shown in Table 2. In most instances, although there was a quantitative change in the level of hydrolysis of specific substrates, there was little evidence to suggest any major difference between the two types of media in the range of substrate preferentially hydrolysed.

The s.e.s of the mean aminopeptidase activity of intracellular enzyme preparations indicated relatively consistent hydrolysis of the substrates for each of the strains examined. The serovars of *L. interrogans* exhibited a definite and consistent range of substrates preferentially hydrolysed. These included the naphthylamide derivatives of alanine, arginine, aspartic acid, glutamine, leucine, lysine, methoxyleucine and ornithine (Table 3). Hydrolysis occurred with other substrates but to a lesser extent. A consistent pattern of preferred substrate hydrolysis was not so readily observed in the results from the saprophytic *L. biflexa* strains, however with the exception of 'semaramga' strains, these strains uniformly showed maximum hydrolysis of the

Table 3. *Comparison of the mean aminopeptidase activities of intracellular extracts from leptospiral strains of L. interrogans and L. biflexa*

Extracts were prepared from cultures grown in medium without serum (see Methods). Enzyme activities were measured after 4 h incubation. Duplicate assays were carried out on two samples taken from each culture. The results are the mean activities of extracts of three individual cultures of each strain; the S.E.s are shown.

Substrate*	Aminopeptidase activity [$\mu\text{mol } \beta\text{-naphthylamine released in}$ 4 h (mg protein) $^{-1}$]	
	<i>L. interrogans</i> (seven strains)	<i>L. biflexa</i> (six strains)
Ala-ONA	144.8 $\pm$ 14.8	105.5 $\pm$ 39.9
Val-ONA	17.4 $\pm$ 3.5	4.2 $\pm$ 1.1
Leu-ONA	168.2 $\pm$ 26.0	27.8 $\pm$ 10.9
Ile-ONA	17.8 $\pm$ 3.5	1.9 $\pm$ 0.6
Phe-ONA	51.5 $\pm$ 11.0	35.7 $\pm$ 13.1
(4-OMe)Leu-ONA	100.4 $\pm$ 17.9	15.0 $\pm$ 3.6
Asp ¹ -ONA	265.0 $\pm$ 66.7	29.9 $\pm$ 7.9
Glu ² -ONA	28.3 $\pm$ 3.2	8.4 $\pm$ 1.8
Lys-ONA	235.0 $\pm$ 34.1	49.6 $\pm$ 12.6
Arg-ONA	179.0 $\pm$ 25.6	53.0 $\pm$ 11.8
Ser-ONA	38.3 $\pm$ 6.6	28.2 $\pm$ 12.8
Tyr-ONA	64.3 $\pm$ 5.3	18.9 $\pm$ 7.9
Trp-ONA	28.6 $\pm$ 3.8	20.5 $\pm$ 6.7
Gly-ONA	21.1 $\pm$ 5.0	47.0 $\pm$ 21.1
Cys-ONA	32.9 $\pm$ 7.7	4.6 $\pm$ 2.1
Met-ONA	49.3 $\pm$ 8.5	34.2 $\pm$ 12.1
His-ONA	32.7 $\pm$ 5.3	13.3 $\pm$ 3.7
Pro-ONA	14.3 $\pm$ 4.2	22.1 $\pm$ 8.6
(4-OH)Pro-ONA	5.1 $\pm$ 1.9	1.9 $\pm$ 0.9
Pyr-ONA†	7.2 $\pm$ 2.3	2.0 $\pm$ 0.6
Orn-ONA	189.7 $\pm$ 18.8	11.2 $\pm$ 5.0
Gln-ONA	254.9 $\pm$ 42.0	39.8 $\pm$ 16.7

* -ONA, β -Naphthylamine derivative.

† Pyr-ONA, Pyrrolidonyl- β -naphthylamide.

alanyl- β -naphthylamine substrate. Pathogenic leptospires in particular require nutritionally complex media for survival and propagation. The significance of many media constituents is, however, somewhat obscure, for example there have been contradictory reports about the necessity of amino acids in leptospiral media (Ono, 1938; Green, 1945; Johnson & Rogers, 1964). Their exact role in leptospiral energy metabolism is unclear, but some amino acids are known to be incorporated into cellular components (Johnson & Rogers, 1964). One possible explanation of the contradictory observations concerning amino acid requirements of leptospires might be the low cell permeability of free amino acids suggested by Johnson & Rogers (1964). If small peptides were therefore preferentially transported, as has been observed in other bacteria (Wahren & Holme, 1973), this could account for the intracellular exopeptidases demonstrated in this study. Thus amino acids, if required, could be made available intracellularly without addition of free amino acids to the media. The ability to consistently demonstrate intracellular aminopeptidase activity, apparently specific for a preferred range of substrates, suggests that such activity is possibly a part of the leptospiral cell metabolism.

Although some variation was exhibited in the level of hydrolysis, even for the preferred substrates of the *L. interrogans* strains, it was observed that the level of leucyl- β -naphthylamine hydrolysis was consistently higher for the parasitic strains than for the saprophytes. This is in agreement with the observations of Burton *et al.* (1970), but it is possibly unwise to place too much emphasis on the significance of leucine aminopeptidase *per se*, as the levels of hydrolysis of

Table 4. Comparison of intracellular aminopeptidase activities of extracts of *L. illini* and *L. parva* sp. nov. using 22 aminoacyl- β -naphthylamide substrates

Extracts were prepared from cultures grown in medium without rabbit serum (see Methods). Enzyme activities were measured after 4 h incubation. Duplicate assays were carried out on two samples taken from each culture. The results are the mean activities of extracts prepared from three individual cultures of each strain; the s.e.s are shown.

Substrate*	Aminopeptidase activity [$\mu\text{mol } \beta\text{-naphthylamine released in}$ 4 h (mg protein) $^{-1}$]	
	<i>L. illini</i>	<i>L. parva</i> sp. nov.
Ala-ONA	95.0 $\pm$ 9.3	0
Val-ONA	7.0 $\pm$ 2.2	0.2 $\pm$ 0.2
Leu-ONA	10.0 $\pm$ 0.8	0.3 $\pm$ 0.3
Ile-ONA	7.6 $\pm$ 0.6	0.7 $\pm$ 0.3
Phe-ONA	10.4 $\pm$ 0.3	0 $\pm$ 0
(4-OMe)Leu-ONA	3.7 $\pm$ 0.2	0.8 $\pm$ 0.3
Asp ¹ -ONA	1.4 $\pm$ 0.7	0
Glu ² -ONA	4.0 $\pm$ 1.1	0.4 $\pm$ 0.2
Lys-ONA	9.0 $\pm$ 0.2	0.3 $\pm$ 0.3
Arg-ONA	5.1 $\pm$ 1.4	0.7 $\pm$ 0.1
Ser-ONA	27.0 $\pm$ 6.5	0.1 $\pm$ 0.1
Tyr-ONA	12.3 $\pm$ 4.1	0.2 $\pm$ 0.2
Trp-ONA	2.6 $\pm$ 1.0	0.3 $\pm$ 0.3
Gly-ONA	187.3 $\pm$ 34.7	0.6 $\pm$ 0.4
Cys-ONA	9.0 $\pm$ 4.0	0
Cys-ONA Met-ONA	14.7 $\pm$ 4.0	3.9 $\pm$ 2.1
His-ONA	26.1 $\pm$ 7.9	0.3 $\pm$ 0.1
Pro-ONA	54.4 $\pm$ 7.9	0.3 $\pm$ 0.3
(4-OH)Pro-ONA	107.2 $\pm$ 27.2	0.1 $\pm$ 0.1
Pyr-ONA†	11.5 $\pm$ 1.7	0.2 $\pm$ 0.1
Orn-ONA	0.2 $\pm$ 0.4	21.7 $\pm$ 2.8
Gln-ONA	2.8 $\pm$ 0.8	0

* -ONA, β -Naphthylamide derivative.

† Pyr-ONA, Pyrrolidonyl- β -naphthylamide.

other substrates were also markedly higher for the parasitic compared to the saprophytic strains (Table 3). Burton *et al.* (1970) examined the aminopeptidase activities of leptospiral strains using only two substrates, and demonstrated a preferential hydrolysis by the saprophytic strains of the alanyl- over the leucyl- β -naphthylamide. The observation has been confirmed in this work (Table 3). Their observed preferential hydrolysis by the pathogenic strains of the leucyl- over alanyl- β -naphthylamide was also shown to be generally valid (Table 3), however it was only confirmed with five of the seven pathogenic strains tested and therefore may be invalid if a larger number of strains was examined.

An association between microbial aminopeptidase activity and pathogenicity has been suggested (Burton *et al.*, 1970; Neill & Ball, 1980; Watson & Perrine, 1975). In this study differences in preferred substrate hydrolysis did exist between the potentially pathogenic *L. interrogans* strains and the saprophytic *L. biflexa* strains, and a most apparent quantitative difference in levels of hydrolysis was demonstrated for the two species (Table 3). It would unfortunately be inappropriate to extrapolate from these observations, as the *L. interrogans* strains employed had been subjected to prolonged passage in laboratory media and possibly might not reflect accurately the qualitative or quantitative substrate profile of fresh isolates. This aspect deserves further study.

Of the 22 substrates used in the assay, preferred hydrolysis by the extract from *Leptospira illini* was in the order glycyl-, hydroxypropyl- and alanyl- β -naphthylamide (Table 4). The hydroxyproline- β -naphthylamide was not significantly hydrolysed by extracts from any of the

other leptospiral strains tested. Similarly, with *Leptospira parva* sp. nov. extracts, only the ornithine substrate was hydrolysed to any extent. It is of interest to note that these two strains presented somewhat unique profiles of hydrolysis amongst the 15 cultures examined. Hovind-Hougen (1979) distinguished *L. illini* from other leptospires on a morphological basis and suggested the creation of the new genus *Leptonema*. The results presented here using only one *L. illini* strain would not conflict with their findings. However, the creation of a new leptospiral species for the organism designated *L. parva* sp. nov. (Hovind-Hougen *et al.*, 1981) appears inappropriate, as the results indicate no similarity with any of the leptospiral strains examined. The observed distinction of these two strains from *Leptospira* is supported to some extent by DNA base composition; leptospiral DNA ranges from 35–41 mol. % G + C whereas *L. parva* and *L. illini* have compositions of 47.4 mol. % G + C and 51–53 mol. % G + C respectively (Hovind-Hougen *et al.*, 1981).

No attempt was made to establish whether the activity exhibited in any extract was from a single non-specific enzyme or from several enzymes specific for individual substrates. The intracellular hydrolytic activity exhibited by the cultures examined indicated greater homogeneity within *L. interrogans* than within *L. biflexa* and illustrated a uniqueness of activity for *L. illini* and *L. parva* sp. nov. It therefore appears that the aminopeptidase profile of *Leptospira* could serve as a taxonomic tool and might prove useful for interspecies and perhaps intraspecies differentiation.

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