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Mammalian Lens Dipeptidyl Aminopeptidase III

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Summary:

An intracellular exopeptidase identified as dipeptidyl aminopeptidase III (DAP III) was found to be abundant in the bovine lens. The enzyme contained in aqueous extracts exhibited a marked preference, compared to other dipeptidyl-β-naphthylamides, for the release of Arg-Arg from Arg-Arg-2-NNap at the optimum pH 9.0 and 37°. The K_m for this substrate was estimated to be 2.83×10^{-5}M. Lens DAP III was inhibited by EDTA, p-chloromercuriphenyl sulfonate, and puromycin. Lens aminopeptidase activities measured at pH 7.5 on the β-naphthylamides of leucine, alanine, and arginine, included for comparison, suggested that not only is leucine aminopeptidase abundant, but also other aminopeptidases that appear to include alanine aminopeptidase and aminopeptidase B.

Introduction:

Dipeptidyl aminopeptidase III (EC 3.4.14-), belongs to a class of enzymes known as dipeptidylpeptide hydrolases. The enzyme was first isolated from bovine anterior pituitary extracts by Ellis and Nuenke (1967). McDonald et al. (1971) and McDonald and Schwabe (1977) have distinquished among the activities of four distinct dipeptidyl aminopeptidases. Their summary of the subcellular distribution of these enzymes indicates that dipeptidyl aminopeptidases I and II are lysosomal, whereas dipeptidyl aminopeptidase III (DAP III) is a cytosol exopeptidase. Typically, DAP III removes NH_2-terminal dipeptides at neutral to alkaline pH with a high degree of specificity for Arg-Arg-β-naphthylamide (Arg-Arg-2-NNap). The unpublished studies of McDonald and Ellis, which have been summarized elsewhere (McDonald and Schwabe, 1977), have shown that red blood cells are a rich source of DAP III, and that this activity is probably responsible for the angiotensinase activity of hemolyzed red cells.

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The present communication reports the presence in the bovine lens of a new peptide hydrolase that is herein characterized as DAP III.

Materials and Methods:

Bovine lenses were acquired from Burbage's Meat Packing Company, Ravenel, South Carolina, within minutes after death. Lenses were homogenized in 4 parts of cold distilled water (20% w/w solution), using a procedure developed by Swanson (1965). Following centrifugation at 20,000 g for 20 min at 4°, a colorless supernatant was removed for the enzyme assays. The protein content of the aqueous extracts was typically about 50 mg per ml. Since the lens has no blood supply, there was no ambiguity regarding the source of the enzyme studied. The direct recording fluorometric assay procedure described by McDonald et al. (1966) was employed for all assays. The activity of the putative DAP III was assayed with Arg-Arg-2-NNap (Bachem Inc. Fine Chemicals, Torrance, Calif). Assay mixtures consisted of 2.7 ml H_2O, 1.0 ml of 0.25 M glycine-NaOH buffer, pH 9.0, and 0.1 ml of bovine lens supernatant. These mixtures were preincubated at 37° for approximately 4 min prior to the addition of 0.2 ml 0.8 mM Arg-Arg-2-NNap in water to initiate the reaction in a final volume of 4 ml.

The reaction mixture was maintained at 37° in a water-jacketted cuvette holder of a Turner fluorometer. The cuvette was continuously irradiated with light at 335 nm and the fluorescence intensity was monitored and recorded with a strip chart recorder. The rate of reaction was indicated by the rate of increase in the intensity of the fluorescence at 410 nm. A standard solution of β-naphthylamine hydrochloride in the assay buffer was used for calibration. Rates of hydrolysis were established by comparing the rate of increase in fluorescence intensity to a standard curve relating fluorescence intensity to β-naphthylamine concentration. One unit of DAP III is defined as the amount of enzyme required to release the β-naphthylamine from Arg-Arg-2-NNap at the rate of 1 μmole per minute under the conditions of the assay.

Aminopeptidase activities were also assayed fluorometrically (McDonald et al., 1964) in reaction mixtures that consisted of 1 ml of 0.25 M Tris-HCl buffer, pH 7.5, 0.1 ml of an appropriate sample of lens supernatant, and water to give a volume of 3 ml. This mixture was preincubated 5 min at 37° prior to the addition of 1 ml of 0.8 mM aminoacyl-β-naphthylamide (pre-warmed to 37°).

Potential inhibitors and activators were tested by incorporating them into the assay mixtures and extending the preincubation period to 30 min. Controls were preincubated for the same period of time.

All the β-naphthylamide substrates were obtained from Bachem Inc. (Torrance, Calif.). The purity and identity of these compounds were established by thin layer chromatography and amino acid analysis. The amount of β-naphthylamine in the acid hydrolysates was established fluorometrically.

Protein concentrations were determined by the procedure of Bradford (1976). Specific activities were expressed as nmoles of Arg-Arg-2-NNap hydrolyzed/min/mg of protein at 37° and pH 9.0.

Results:

Table I presents the rates of hydrolysis by bovine lens supernatants of a variety of dipeptidyl-β-naphthylamides and, for comparison, a few

TABLE I

Specificity of Dipeptidyl-β-Naphthylamide Hydrolysis by DAP III and, for Comparison, Some Aminoacyl-β-Naphthylamides by Aminopeptidases in the Lens Supernatant

Substrate	Activity	
β-naphthylamide	DAP III	
	Specific n moles min^{-1} mg^{-1} protein	Relative % (relative to Arg-Arg-2-NNap)
Arg-Arg	1.81	100
Cbz-Arg-Arg	0	0
Leu-Ala	0.50	30
Ile-Ala	0.15	8.3
Ala-Ala	0.12	6.6
Lys-Ala	0.058	3.2
Gly-Phe	0.06	3.3
Gly-Arg	0.012	0.66
Arg-Ala	0.004	0.22
Arg-Pro	0.003	0.17
	Aminopeptidase	
Ala	2.69	149%
Lys	2.34	129
Met	1.97	109
Leu	1.84	102

selected aminoacyl-β-naphthylamides. Like the DAP III purified from bovine pituitary tissue (Ellis and Nuenke, 1967), the lens enzyme exhibited a marked preference for the hydrolysis of Arg-Arg-2-NNap.

The initial rates of hydrolysis of the dipeptidyl-β-naphthylamides were linear from the point of substrate addition, showing that the β-

naphthylamine liberated in the first few minutes of the reaction was not, to any significant degree, the result of the stepwise removal of the amino acids. The DAP III character of the lens enzyme was revealed by its lack of activity on the benzyloxycarbonyl (Cbz) derivative of the same substrate. The aminoacyl-β-naphthylamides were cleaved non-specifically at a faster rate, presumably by the preponderance of leucine aminopeptidase (LAP) that is known to be present in the bovine lens (Hanson, et al., 1965 and Thompson and Carpenter, 1976). The relatively high rates of hydrolysis of the β-naphthylamides of lysine, methionine, and alanine were indicative of the presence of other aminopeptidases with specificities resembling aminopeptidase B (McDonald et al., 1964 and McDonald and Schwabe, 1977) and alanine aminopeptidase (McDonald and Schwabe, 1977). Considerable hydrolysis was observed on Leu-Ala-2-NNap. Very low or negligible activities were observed on the β-naphthylamide derivatives of Arg-Ala, Gly-Arg and Arg-Pro.

Figure 1 illustrates the effect of pH on lens supernatant DAP III and aminopeptidase with Arg-Arg-2-NNap and Leu-2-NNap, respectively. DAP III exhibited an optimum pH at 9.0 and aminopeptidase (leucyl arylamidase) at pH 7.5 to 8.0.

The effect of temperature on the enzymatic hydrolysis of Arg-Arg-2-NNap by lens supernatant DAP III at pH 9.0 is presented in Table II. The heat stability of DAP III was assessed by increasing the time and temperature of preincubation (pH 9.0) in the assay system. DAP III activity was stable at temperatures of 37° to 40° then decreased to 81% at 45°, to 18% at 50°, and was almost completely inactivated at 55°.

The rate response to substrate concentration for the hydrolysis of Arg-Arg-2-NNap by the lens supernatant is illustrated in Fig. 2. The Lineweaver-Burk plot is shown in the inset. A Km of 2.83 x 10^{-5}M was calculated for the hydrolysis of Arg-Arg-2-NNap at pH 9.0 and 37°.

The effect of a number of inhibitors is shown in Table III for both DAP III and aminopeptidase. The hydrolysis of Arg-Arg-2-NNap by lens

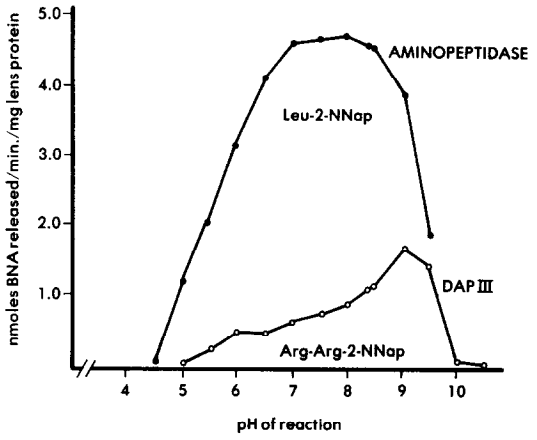

Fig. 1. Effect of pH on hydrolysis rates for the DAP III and aminopeptidase (leucyl arylamidase) of bovine lens. Buffers and range include the following: 60 mM Na acetate pH 4.0 to 6.0, 50 mM Na phosphate pH 6.0 to 7.5, 60 mM Tris-HCl pH 7.0 to 8.5, and 60 mM glycine-NaOH pH 8.4 to 10.5.

TABLE II

THE EFFECT OF HEAT TREATMENT AT pH 9.0 ON THE STABILITY OF THE DAP III ACTIVITY CONTAINED IN THE LENS EXTRACT

Temperature (C°)	Time (Minutes)	Activity	
		Arg-Arg-2-NNap Specific Activity (nmoles min^{-1} mg^{-1} protein)	Relative (%)
37°	4	1.78	100
	15	1.85	104
	30	1.76	99
	60	1.64	92
	90	1.67	94
40°	4	1.88	106
45°	4	1.44	81
50°	4	0.32	18
55°	4	0.047	2.6

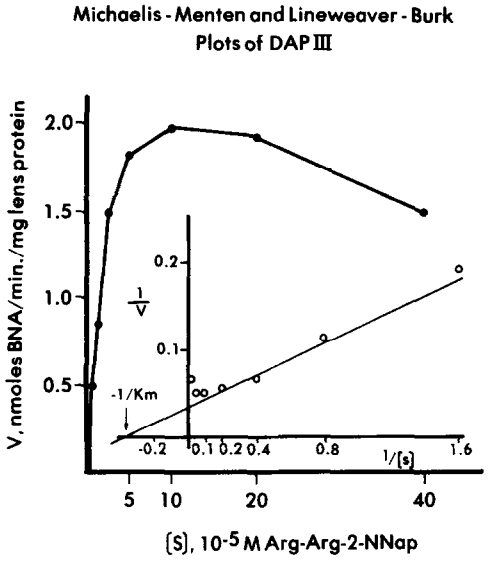

Fig. 2. Rate response to substrate concentration for the hydrolysis of Arg-Arg-2NNap at pH 9.0 and 37° by DAP III from bovine lens supernatant. Inset shows Lineweaver-Burk plot.

supernatant DAP III was completely inhibited by EDTA, p-chloromercuriphenyl sulfonate (PCMS), and puromycin. A partial restoration of activity was noted when 2-mercaptoethanol was added to the PCMS-inhibited enzyme at a concentration of 7 mM, which was a 70-fold excess over the PCMS. When 2-mercaptoethanol alone was used, approximately 50% inhibition was observed.

The aminopeptidase activity of the lens supernatant showed little or no inhibition of Leu-2-NNap hydrolysis following treatment with 2-mercaptoethanol and EDTA. However, p-chloromercuriphenyl sulfonate, Zn acetate, and puromycin were strongly inhibitory.

Discussion:

These studies have demonstrated the presence within bovine lens tissue extracts of a dipeptidyl aminopeptidase III which, like the pituitary enzyme described by Ellis and Nuenke (1967), exhibited a high degree of specificity for the hydrolysis of Arg-Arg-2-NNap. Lens DAP III further resembled the pituitary enzyme with respect to its pH optimum (pH 9.0) and its susceptibility to substrate inhibition when the concentration of Arg-Arg-2-NNap

TABLE III

The Effect of Inhibitors on DAP III and Aminopeptidase Activities in the Lens Supernatant

Inhibitor and Concentration	Time Minutes (Preincubation)	Rate of Hydrolysis (nmoles min^{-1} mg^{-1} protein)		Inhibition (%)	
		DAP III Arg-Arg-2-NNap	Aminopeptidase Leu-2-NNap	DAP III	Amino-peptidase
None	4	1.80	7.2	0	0
"	30	2.55		0	
1mM EDTA	30	0	6.6	100	8
1mM EDTA + 2mM Zn $(AC)_2$†	4	0.81		55.3	
1mM EDTA + 2mM Zn $(AC)_2$†	30	1.0		44.7	
2mM Zn $(AC)_2$	4	1.0	0	43.2	100
1.0 mM PCMS*	4	0	0.17	100	95
" "	30	0		100	
0.1 mM PCMS & 7mM MCE*†	4	0.345	2.83	80	16
" "	30	1.2		35.2	
7mM MCE	4	0.81	7.2	55.3	0
"	30	1.0		44.5	
2mM puromycin	30	0	0	100	100

*p-chloromercuriphenyl sulfonate (PCMS), 2-mercaptoethanol (MCE), acetate (AC).

† The second additive was introduced following incubation with the first.

exceeded 0.1 mM. The purified pituitary enzyme showed inhibition when the concentration exceeded 0.05 mM. Whereas the pituitary enzyme was reported to have no activity on Leu-Ala-2-NNap, the lens extract hydrolyzed this substrate at a substantial rate (30% of Arg-Arg-2-NNap). However, meaningful comparisons will have to await the purification of lens DAP III.

Bovine lens tissue is a rich source of leucine aminopeptidase (EC-3·4·11.1) that has been used by Hanson et al. (1965) for their preparation of the crystalline enzyme. In keeping with these findings, our bovine lens extracts showed a pronounced rate of hydrolysis on Leu-2-NNap at pH 7.5. However, unlike leucine aminopeptidase purified from bovine lens (Hanson et al. 1967) and porcine kidney (Hanson et al., 1967 and McDonald et al., 1964), both of which were shown to hydrolyze Leu-2-NNap at much greater rates than the alanine and lysine derivatives, the unfractionated lens supernatant used in this study exhibited greater rates of hydrolysis on the alanine and lysine derivatives. On this basis it would appear that the bovine lens supernatant is relatively rich in other aminopeptidases. Likely candidates include alanine aminopeptidase and aminopeptidase B, the properties of which have been summarized by McDonald and Schwabe (1977).

Although we cannot assign a specific function to dipeptidyl amino-peptidase III in lens tissue, it is tempting to equate proteolytic enzyme activities with structural changes in lens proteins. Accordingly, further efforts will be made to purify the enzyme from lenses and define more precisely the possible function of this exopeptidase on α, β and γ crystallin degradation in cataractous and aging human lenses.

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