BBA 66344

A SOLUBLE ASPARTATE AMINOPEPTIDASE FROM DOG KIDNEY

H. S. CHEUNG AND D. W. CUSHMAN
Squibb Institute for Medical Research, New Brunswick, N.J. 08903 (U.S.A.)

(Received February 8th, 1971)

SUMMARY

A soluble, Mn^{2+}-activated aspartate aminopeptidase has been purified 33-fold from dog kidney. The purified enzyme is free of other aminopeptidases, including the particulate, Ca^{2+}-stimulated glutamate aminopeptidase, aminopeptidase A. Aspartate aminopeptidase is neither stimulated by Ca^{2+} nor inhibited by EDTA, but is activated by preincubation with $MnCl_2$. It hydrolyzes aspartyl-β-naphthylamide 3 times more rapidly than glutamyl-β-naphthylamide, and does not hydrolyze other amino acyl-β-naphthylamides. It does not cleave the N-terminal asparagine residue from [Asn^1,Val^5]-angiotensin II, but rapidly and quantitatively cleaves the N-terminal aspartic acid residue from [Asp^1,Ile^5]-angiotensin I. Because of its unique specificity, aspartate aminopeptidase may play a physiological role as an angiotensinase.

Mammalian tissues contain aminopeptidases or "arylamidases" with a great variety of substrate specificities. Enzymes have been isolated that preferentially hydrolyze peptide bonds involving N-terminal acidic amino acids (glutamate or aspartate)[1,2], basic amino acids (lysine or arginine)[3], alanine[4–6], methionine[7,8], glycine[9], cystine[10], β-aspartate[11], or pyrrolidone carboxylate[12]. One such enzyme, aminopeptidase A[1,2], is a Ca^{2+}-stimulated enzyme of plasma and kidney microsomes that hydrolyzes glutamyl-β-naphthylamide (GluNA) more rapidly than it does aspartyl-β-naphthylamide (AspNA); it is responsible for the rapid destruction of angiotensin II in plasma by hydrolytic removal of the N-terminal aspartic acid residue[13,14]. In the course of studying such "angiotensinase" activity of dog kidney extract, we have found a second aminopeptidase capable of cleaving the N-terminal aspartate residue from angiotensin, but which is more active on AspNA than on GluNA. This new, soluble, Mn^{2+}-activated enzyme, aspartate aminopeptidase, has been separated from other kidney aminopeptidases and shown to be different from aminopeptidase A.

Aspartate aminopeptidase activity was assayed by spectrophotometric determination of β-naphthylamine released from the substrate, AspNA[15]. The enzyme was first activated by incubation for 1 h at 30° with 50 mM $MnCl_2$ (Fig. 1), and then incubated for 90 min at 30° in a 3-ml assay mixture containing 0.75 mM AspNA and 100 mM Tris–HCl (pH 7.5). Enzymic hydrolyses of other amino acyl-β-naphthyl-

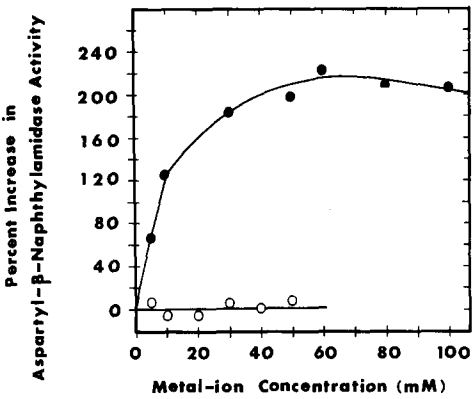

Fig. 1. Effect of Mn^{2+} and Ca^{2+} on aspartate aminopeptidase activity. Enzyme purified through the second $(NH_4)_2SO_4$ stage (0.56 mg protein) was preincubated for 60 min with $MnCl_2$ (●) or $CaCl_2$ (○) at the indicated final concentrations, and then assayed for aspartyl-β-naphthylamidase activity, as described in the text.

amides were assayed under identical conditions, except where indicated in the text. A unit of naphthylamidase activity is the amount catalyzing the formation of 1 μmole of β-naphthylamine per min. Protein was determined by the biuret method[16] or, after chromatographic purification of the enzyme, spectrophotometrically[17].

Aspartate aminopeptidase was first detected during a study of the effect of metal ions on aspartyl-β-naphthylamidase and glutamyl-β-naphthylamidase activities of "microsomal" and soluble fractions of dog kidney extract (Table I). Both naphthylamidase activities in all fractions were stimulated by added Ca^{2+}, as would be expected for aminopeptidase A[1,], but only aspartyl-β-naphthylamidase activity was increased by preincubation with Mn^{2+}. About half of the Ca^{2+}-stimulated glutamyl-β-naphthylamidase activity (aminopeptidase A) was found, as expected, in the "microsomal" fraction, but more than 80% of the Mn^{2+}-activated aspartyl-β-naphthylamidase activity was soluble. Although separation of the two enzymes was incomplete, the results suggested the co-existence in dog kidney extract of a soluble,

TABLE I

DISTRIBUTION OF ASPARTYL- AND GLUTAMYL-β-NAPHTHYLAMIDASE ACTIVITIES IN MICROSOMAL AND SOLUBLE FRACTIONS OF DOG KIDNEY HOMOGENATE

5 g of dog kidney was homogenized for 2 min with 50 ml of 50 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose, using a motor-driven teflon pestle. The homogenate was centrifuged for 40 min at 30 000 × *g*, and the resulting supernate for 2 h at 105 000 × *g*. Assays with Mn^{2+}-preincubated enzyme were performed as described in the text; other assays were carried out without preincubation, either without additions or with the addition of 50 mM $CaCl_2$.

Fraction	*Naphthylamidase activity (munits)*					
	AspNA			*GluNA*		
	No metal	*+MnCl₂*	*+CaCl₂*	*No metal*	*+MnCl₂*	*+CaCl₂*
Dog kidney extract	13.4	40.5	—	49.4	25.3	—
Microsomal fraction	2.7	6.5	14.3	12.6	5.2	52.8
Soluble fraction	11.3	30.6	20.9	28.4	20.6	67.5

Mn^{2+}-activated aspartyl-β-naphthylamidase (aspartate aminopeptidase) and of a particulate, Ca^{2+}-stimulated glutamyl-β-naphthylamidase, presumed to be aminopeptidase A.

In dog kidney homogenates, enzymes catalyzing the hydrolysis of arginyl-β-naphthylamide (ArgNA) and leucyl-β-naphthylamide (LeuNA) are 15 and 30 times more active, respectively, than that cleaving AspNA; it is necessary to show that the Mn^{2+}-activated aspartyl-β-naphthylamidase activity is neither a property of one of these enzymes nor of aminopeptidase A. The aspartyl-β-naphthylamidase activity was distributed quite differently into $(NH_4)_2SO_4$ fractions than were other naphthylamidase activities; only 10% of the aspartyl-β-naphthylamidase activity was precipitated in the range of 40–100% saturation $(NH_4)_2SO_4$, whereas 55% of the glutamyl-β-naphthylamidase or leucyl-β-naphthylamidase and 45% of the arginyl-β-naphthylamidase activities were precipitated between these limits.

TABLE II

PARTIAL PURIFICATION OF ASPARTATE AMINOPEPTIDASE FROM DOG KIDNEY

Naphthylamidase activities were assayed as described in the text, after preincubating fractions for 1 h at 30° with 50 mM $MnCl_2$ (Fractions 1–4) or 300 mM $MnCl_2$ (Fraction 5).

Fractionation step	*Total protein (mg)*	*Total activity (munits)*	*Specific activity (munits/mg)*	*Activity recovered (%)*	*Purification (-fold)*	*Specificity*		
						LeuNA/AspNA	*ArgNA/AspNA*	*GluNA/AspNA*
Dog kidney extract	19 950	4 680	0.24	100	1	14	33	1.1
30–40% $(NH_4)_2SO_4$ (1)	3 010	3 540	1.18	76	5	1.3	3.3	0.30
30–40% $(NH_4)_2SO_4$ (2)	780	2 410	3.10	52	13	0.6	2.0	0.27
DEAE-cellulose	300	1 480	4.95	32	21	0	0	0.34
BioGel P-300	120	910	7.80	20	33	0	0	0.36

Further purification of the aspartyl-β-naphthylamidase activity of dog kidney extract effected the removal of arginyl-β-naphthylamidase and leucyl-β-naphthylamidase activities (Table II); the GluNA/AspNA activity ratio was not changed in the last three purification steps, suggesting that the residual hydrolysis of GluNA was a property of aspartate aminopeptidase. For the purification outlined in Table II, 300 g of dog kidney tissue was blended with 900 ml of 50 mM Tris–HCl (pH 7.5) and centrifuged for 30 min at 30 000 × *g*. The supernatant was fractionated twice by precipitation between the limits of 30 and 40% saturated $(NH_4)_2SO_4$ (16 to 22 g/100 ml), using $(NH_4)_2SO_4$ containing 2% $(NH_4)_2CO_3$ (pH 7.5). The second $(NH_4)_2SO_4$ fraction was dialyzed and chromatographed on a 1.8 cm × 13-cm column of Whatman DE52 microgranular DEAE-cellulose, developed with a linear gradient of 0–400 mM KCl in 50 mM Tris–HCl (pH 7.5). Activity eluted between 80 and 150 mM KCl was concentrated by $(NH_4)_2SO_4$ precipitation and further purified by gel permeation chromatography on a 2 cm × 45-cm column of BioGel P-300; the high-molecular-weight enzyme was only slightly retarded on this column. An overall purification of 33-fold was achieved. The poor recoveries of activity in the last two steps might have been due to incomplete activation. The amount of Mn^{2+} required to give maximal activation of the crude enzyme was increased from 50 mM (Fig. 1)

to as high as 300 mM, even with increased times of preincubation. This problem has not been studied further.

Aspartate aminopeptidase does not hydrolyze β-naphthylamide derivatives of amino acids, other than aspartic and glutamic acids. That this specificity extends to hydrolysis of true peptide bonds is indicated by the action of the 33-fold purified enzyme on two synthetic angiotensins: [Asn^1,Val^5]-angiotensin II was not hydrolyzed after prolonged incubation with the enzyme, whereas [Asp^1,Ile^5]-angiotensin I was rapidly cleaved to yield aspartic acid and the nonapeptide, Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu, which was not further degraded. It is probable that most so-called "arylamidases" or "naphthylamidases" will prove to be true aminopeptidases when tested for hydrolytic activity on peptide substrates containing three or more amino acid residues.

The only aminopeptidase previously reported to be specific for dicarboxylic amino acids is aminopeptidase A, first described by GLENNER *et al.*[1]. Several lines of evidence indicate that the aspartate aminopeptidase described in this paper is a new enzyme, not identical with aminopeptidase A. Aspartate aminopeptidase is a soluble enzyme activated by incubation with Mn^{2+}, but unaffected by Ca^{2+} or EDTA; aminopeptidase A, on the other hand, is a microsomal enzyme that is stimulated by Ca^{2+} and inhibited by EDTA or Mn^{2+}. Aspartate aminopeptidase is 3 times more active on AspNA than on GluNA, whereas aminopeptidase A is a glutamate aminopeptidase, 6 times more active on GluNA than on AspNA[1]. Both aminopeptidases were optimally active at pH 7.5. Aspartate aminopeptidase has not been demonstrated in tissues other than the kidney, but, where present in high concentration, it could serve an important physiological role as an "angiotensinase".

REFERENCES

1 G. G. GLENNER, P. J. MCMILLAN AND J. E. FOLK, *Nature*, 194 (1962) 867.
2 I. NAGATSU, T. NAGATSU, T. YAMAMOTO, G. G. GLENNER AND J. W. MEHL, *Biochim. Biophys. Acta*, 198 (1970) 255.
3 V. K. HOPSU, K. K. MÄKINEN AND G. G. GLENNER, *Arch. Biochem. Biophys.*, 114 (1966) 557, 567.
4 N. REHFELD, J. E. PETERS, H. GIESECKE, L. BEIER AND R. J. HASCHEN, *Acta Biol. Med. Ger.*, 19 (1967) 809.
5 F. J. BEHAL AND G. H. LITTLE, *Clin. Chim. Acta*, 21 (1968) 347.
6 F. J. BEHAL, G. H. LITTLE AND R. A. KLEIN, *Biochim. Biophys. Acta*, 178 (1969) 118.
7 F. J. BEHAL AND M. N. STORY, *Arch. Biochem. Biophys.*, 131 (1969) 74.
8 P.-L. MÄKINEN AND J. RAEKALLIO, *Acta Chem. Scand.*, 22 (1968) 597.
9 A. S. BRECHER AND J. B. SUSZKIW, *Biochem. J.*, 112 (1969) 335.
10 I. SJÖHOLM, *FEBS Letters*, 4 (1969) 135.
11 F. E. DORER, E. E. HALEY AND D. L. BUCHANAN, *Arch. Biochem. Biophys.*, 127 (1968) 490.
12 R. W. ARMENTROUT, *Biochim. Biophys. Acta*, 191 (1969) 756.
13 P. A. KHAIRALLAH, F. M. BUMPUS, I. H. PAGE AND R. R. SMEBY, *Science*, 140 (1963) 672.
14 D. REGOLI, B. RINIKER AND H. BRUNNER, *Biochem. Pharmacol.*, 12 (1963) 637.
15 I. NAGATSU, L. GILLESPIE, J. M. GEORGE, J. E. FOLK AND G. G. GLENNER, *Biochem. Pharmacol.*, 14 (1965) 853.
16 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
17 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.