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N^{ϵ}-(β-ASPARTYL)LYSINURIA IN CHILDREN WITH VARIOUS PATHOLOGICAL CONDITIONS

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Summary

The isolation, identification, and quantitative determination of an unusual urinary dipeptide, N^{ϵ}-(β-aspartyl)lysine, is described, as well as its synthesis. This compound was observed in children in various disease states, but without any correlation with a particular symptom. Its origin is discussed.

Introduction

Whilst screening for aminoacidopathies by two-dimensional thin-layer chromatography, we recently discovered a new amino acid, which turned out to be N^{ϵ}-(carboxymethyl)lysine (N^{ϵ}-CM-Lys) [1]. Afterwards we observed that this spot was not always homogeneous and apparently might also represent another compound. This second compound was no longer detectable after acid hydrolysis and thus might be a small peptide. The constant excretion of relatively large amounts of this ninhydrin-positive substance in one of our patients prompted us to investigate its structure, which appeared to be N^{ϵ}-(β-aspartyl)-lysine (N^{ϵ}-(β-Asp)Lys). Frimpter [2] found this isopeptide in the urine of a patient and thought it was connected with a new syndrome. We considered it useful to investigate whether this compound could be linked to a particular disease or symptom and we therefore analysed a number of urine samples from patients with various pathological conditions excreting N^{ϵ}-(β-Asp)Lys. In this paper we describe the isolation, identification and quantitative determination of the isopeptide. Speculations about its origin and biological significance will be given.

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Materials and methods

1. Reference commercial dipeptides

L-Lysyl-L-aspartic acid was obtained from Sigma Chemical Company, St. Louis, Miss., U.S.A. and N^{α}-(α-L-aspartyl)-L-lysine and N^{α}-(β-L-asparty l)-L-lysine from Bachem Feinchemikalien AG, Liestal, Schweiz.

2. Synthesis of N^{ϵ}-(β-L-asparty l)-L-lysine and N^{ϵ}-(α-L-asparty l)-L-lysine

tert.-Butyloxycarbonyl-L-aspartic anhydride (5 mmol) [3,4] was added to a mixture of N^{α}-*tert.*-butyloxycarbonyl-L-lysine (5 mmol) [5] and triethylamine (10 mmol) in 20 ml of acetonitril/water (4 : 1, v/v). The solution was kept at room temperature for 1.5 h and then 100 ml of H_2O was added, followed by acidification with 2 M $KHSO_4$ to pH 2. The acidic solution was extracted four times with 50 ml of ethylacetate and the combined organic layer dried over Na_2SO_4. Ethylacetate was evaporated and the residue was dissolved in 20 ml of 3 M HCl in ethylacetate.

A precipitate was formed immediately. After 0.5 h at room temperature the solvent was evaporated under reduced pressure and the white powder was kept over KOH in a vacuum desiccator. Ion-exchange column chromatography showed that the material contained nearly equal amounts of N^{ϵ}-(β-Asp)Lys and N^{ϵ}-(α-Asp)Lys and traces of lysine and aspartic acid. The two peptides were separated by column chromatography on Aminex Q-150 S, H^+-form (column dimensions: 100 cm × 2.5 cm). The column was loaded with portions of 200 mg of the peptide mixture and eluted with 0.1 M pyridine/acetic acid, pH 3.65 at a flow rate of 7.3 ml/min at 50° C. The fractions between 1200 and 1500 ml of the eluate contained N^{ϵ}-(β-Asp)Lys.

Subsequently N^{ϵ}-(α-Asp)Lys was eluted from the column with 1 M pyridine. It was present in the fractions between 500 and 600 ml of the eluate. Both peptides were crystallized from water/ethanol.

N^{ϵ}-(β-Asp)Lys: 300 mg. $[\alpha]_D^{20} = +1.5°$ (*c* 0.6; H_2O). $C_{10}H_{19}N_3O_5$, calc.: C 45.98, H 7.28, N 16.09, O 30.65; found: C 45.54, H 7.40, N 15.82, O 30.79.

N^{ϵ}-(α-Asp)Lys: 225 mg. $[\alpha]_D^{20} = +5.7°$ (*c* 0.3; H_2O). $C_{10}H_{19}N_3O_5$, calc.: C 45.98, H 7.28, N 16.09, O 30.65; found: C 45.76, H 7.42, N 16.07, O 30.33.

3. Isolation and identification of N^{ϵ}-(β-asparty l)lysine

Urine (500 ml) of index patient T.S. was filtered and then applied to a column of Dowex 50W × 8, 50—100 mesh, H^+-form (19 cm × 4 cm). The resin was washed with 2 l of H_2O and the isopeptide-containing fraction eluted with 1 l of 1 M pyridine (the eluate was monitored for the presence of the isopeptide by thin—layer chromatography). After evaporation of the solvent the residue was dissolved in 10 ml of H_2O. Portions of 1 ml were fractionated on a column of Aminex-MS, fraction B, H^+-form (106 cm × 2.5 cm) at a flow rate of 6.3 ml/min at 50° C. The elution was performed with 0.1 M pyridine/acetic acid, pH 3.5. The isopeptide was found in the fractions between 2100 and 2500 ml of the eluate. The material obtained from the ten 1-ml portions was pooled and subjected once more to chromatography on Aminex (flow rate: 5.9 ml/min; temperature: 50° C; solvent: 0.1 M pyridine/acetic acid, pH 3.4). The isopeptide was present in the fractions between 3200 and 3400 ml of the eluate and

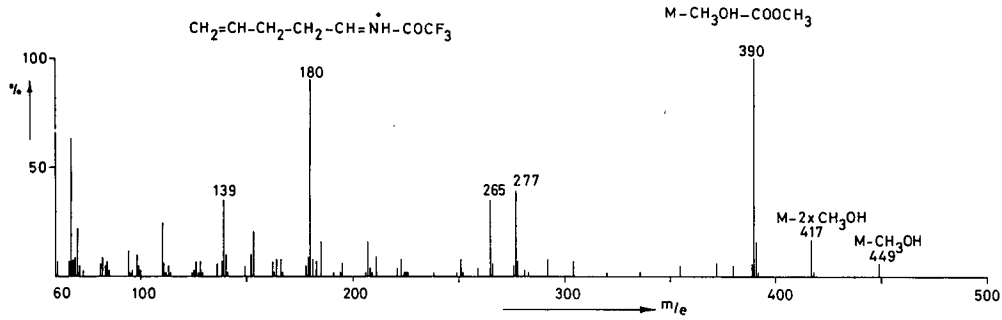

Fig. 1. Mass spectrum of N^{ϵ}-(β-aspartyl)lysine (*N*-trifluoroacetyl peptide methyl ester), M^{+} = 481.

proved to be pure by thin-layer chromatography. The total yield was about 4 mg.

The structure of the isopeptide was proved as follows: Hydrolysis in 6 M HCl for 24 h at 110° C yielded equimolar amounts of aspartic acid and lysine.

The mass spectrum of the *N*-trifluoroacetyl (*N*-TFA) methyl ester derivative of the substance indicated a dipeptidic structure, built up from aspartic acid and lysine, with a molecular weight of 481 (Fig. 1) [6]. The spectrum was identical to that of the *N*-TFA methyl ester of N^{ϵ}-(β-Asp)Lys, but differed greatly from those of the *N*-TFA methyl esters of Lys-Asp, N^{α}-(β-Asp)Lys and N^{α}-(α-Asp)Lys. It has to be noted that derivatization of N^{ϵ}-(α-Asp)Lys led to an as-yet-unidentified product, probably with a molecular weight of 559. For this reason the structure of N^{ϵ}-(α-Asp)Lys was verified using the *N*-ethoxycarbonyl methyl ester derivative [7].

Column chromatography of the isopeptide using the TSM 1 standard procedure for physiological fluids showed the same relative retention time (0.97) with regard to norleucine as N^{ϵ}-(β-Asp)Lys. The data for the other peptides are: Lys-Asp 1.50, N^{α}-(α-Asp)Lys 1.35, N^{α}-(β-Asp)Lys 1.14, and N^{ϵ}-(α-Asp)-Lys 1.08.

The presence of a β-aspartyl residue in the isopeptide was also confirmed by two ninhydrin colour reactions, described in literature for the differentiation between α- and β-aspartyl peptides [1]. After paper chromatography of the five synthetic and the isolated dipeptides in the solvent system *n*-butanol/acetic acid/pyridine/water (30 : 6 : 20 : 24, v/v), the spots were visualized by spraying with 1% ninhydrin in ethanol and heating at 110° C for 10 min [8—10]. α-Aspartyl peptides gave rise to violet spots and β-aspartyl peptides, including the isolated one, greyish-blue spots [2]. Incubation of 0.2 μmol of peptide with 20 μl of 1% ninhydrin in 2.5% citrate buffer, pH 2.52, at 37° C for 6 h yielded no colour for the α-aspartyl peptides and a brown colour for the β-aspartyl peptides including the isolated one [10].

4. Thin-layer chromatography

Micro-scale two-dimensional chromatography on 5 cm × 5 cm thin-layer chromatograms of urinary amino acids was performed as described previously [1]. Solvents were: 1, *n*-butanol/pyridine/water (1 : 1 : 1, v/v) and 2, 88% phenol/25% ammonia/water (10 : 0.05 : 1, v/v).

5. Column chromatography

The use of the Technicon TSM 1 standard procedure for physiological fluids revealed that N^{ϵ}-(β-Asp)Lys, isoleucine and N^{ϵ}-CM-Lys were not separated. Therefore a special procedure, separating N^{ϵ}-(β-Asp)Lys from the other amino acids, was developed for the quantitative determination of the isopeptide. Ion-exchange chromatography was performed on a column of Chromobeads C_3 (69 cm × 0.5 cm) at a flow rate of 0.45 ml/min at 55° C. The elution was carried out with a buffer containing 0.05 M sodium citrate and 0.05 M NaOH, adjusted to pH 3.68 with 6 M HCl, for 70 min.

6. Preparation of N-trifluoroacetyl peptide methyl esters

The peptide (1 mg) was dissolved in 1 ml of methanolic 3 M HCl and heated for 3 h at 100° C. The solution was evaporated in vacuo; traces of HCl were removed by co-evaporation with methanol. Subsequently, 1 ml of trifluoroacetic anhydride was added and the mixture kept for 18 h at room temperature. The anhydride was evaporated under a stream of nitrogen and the residue was dissolved in ethylacetate.

7. Gas-liquid chromatography

Gas-liquid chromatography of *N*-trifluoroacetyl peptide methyl esters was carried out on a Varian aerograph 2740-30-01 gas chromatograph, equipped with a dual-flame ionization detector and glass columns (2.00 m × 4 mm, I.D.) packed with 3.8% SE-30 on Chromosorb W/AW-DMCS, HP, 80—100 mesh. The injection port temperature, detector temperature and oven temperature were 200° C, 220° C, and 205° C, respectively. The gas flow rate for nitrogen was 40 ml/min.

8. Mass spectrometry

The 70 eV mass spectra of the *N*-trifluoroacetyl peptide methyl esters were recorded on a Jeol JGC-20 K/JMS-D100 W-JMA combination at a column oven temperature of 230° C, an ion source temperature of 150° C, an accelerating voltage of 3 kV and an ionizing current of 300 μA. As column material, 3.8% SE-30 on Chromosorb W/AW-DMCS, HP, 80—100 mesh was used.

Patients studied

The index patient T.S. was born at term (birth weight 3020 g). The mother suffered from influenza in the fourth month of pregnancy. The father's father had one clubfoot. The patient was admitted to a hospital on the first day of life. The following observations were made: cyanosis, asymmetrical face, underdeveloped right upper limb, clubfeet, right sided hip dislocation, cleft palate. Routine laboratory investigations were negative. The EEG was strongly abnormal. Hydrocephaly developed and grew worse during life (head circumference 91 cm in the terminal stage). He died at the age of 2.5 years.

Other patients

The other patients mentioned in this article are children in whom an inborn error of amino acid metabolism was suspected as judged by the clinical symp-

toms. Inclusion of samples in this study was based on the intensity of the spot of N^{ϵ}-(β-Asp)Lys relative to those of the other amino acids.

Results

In Fig. 2a the two-dimensional thin-layer chromatogram of the urinary amino acids of patient M.v.B. is given. The spot corresponding with N^{ϵ}-(β-Asp)-Lys has been indicated with an arrow. Fig. 2b and Fig. 2c show additions to the urine sample of the synthetic N^{ϵ}-(β-Asp)Lys and a mixture of synthetic N^{ϵ}-(β-Asp)Lys and N^{ϵ}-CM-Lys, respectively. Excretory values of N^{ϵ}-(β-Asp)Lys in ten patients, including T.S., are given in Table I. These were estimated to be the relatively highest excretions observed in 2000 patients as judged by visual inspection of the two-dimensional chromatograms. Also values for N^{ϵ}-CM-Lys are given. No abnormal excretions of other amino acids were present. Only in two patients a diagnosis could be made. M.v.B. had the so-called Menkes Kinky hair syndrome and M.d.V. suffered from cystic fibrosis.

From Table I it can be seen that the presence of N^{ϵ}-(β-Asp)Lys cannot be correlated with a consistent clinical picture. It also appeared that index patient T.S. did not have the highest excretions. In patient I.D. N^{ϵ}-(β-Asp)Lys practi-

TABLE I

Patients	Sex	Age (years)	Urine (ml)	Creatinine (mg/l)	N^{ϵ}-(β-Asp)-Lys (mg/l)	N^{ϵ}-CM-Lys (mg/l)	Diagnosis
Index patient T.S.	m	1 1/12	530	170	18	n.d. **	Multiple congenital defects; hydrocephalus; 2 6/12 †
		1 3/12	530	185	16	n.d.	
		1 7/12	450	245	22	3	
Other patients							
M.v.B.	m	2 2/12	450	390	28	3	Kinky hair syndrome
		2 3/12	610	320	22	2	
A.R.	m	1/12	150 *	80	3	3	Psychomotor retardation; convulsions; microcephaly; perinatal damage
		2	370	520	49	4	
		2 2/12	105	1000	74	6	
I.D.	f	5/12	185 *	155	8	3	Diarrhoea; hyperpyrexia
		6/12	165 *	155	trace	2	
B.J.	m	2/12	300	190	11	2	Dyspepsia; dystrophy
A.E.	f	4/12	230	130	6	6	Psychomotor retardation; gastroenteritis
D.L.	m	9/12	400	240	14	trace	Familial hypotrophy; megacephaly; motor retardation; delayed bone formation
M.d.V.	m	3/12	240	185	9	3	Mucoviscoidosis
M.W.	m	2 6/12	310	610	6	trace	Severe psychomotor retardation
E.v.d.F.	m	8 10/12	750	935	4	5	Hepatosplenomegaly

* 12-h urines; the other samples are 24-h urines.

** n.d., not detected.

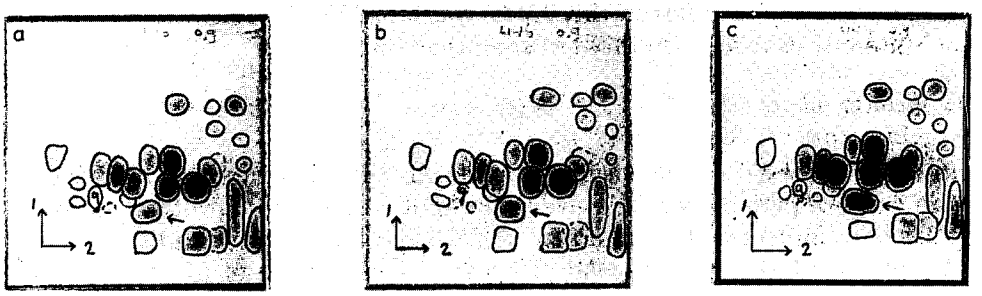

Fig. 2. (a) Unknown amino acid on a two-dimensional thin-layer chromatogram (5 × 5 cm) of urinary amino acids in patient M.v.B. (b) N^{ϵ}-(β-Aspartyl)lysine added. (c) N^{ϵ}-(β-Aspartyl)lysine and N^{ϵ}-(carboxymethyl)lysine added.

cally disappeared from the urine in one month. In most patients excretions of N^{ϵ}-CM-Lys were lower than those of N^{ϵ}-(β-Asp)Lys. The position of N^{α}-(β-Asp)Lys is very near to the spot of N^{ϵ}-(β-Asp)Lys on the two-dimensional chromatogram. Its velocity is somewhat lower in both solvents. During this investigation we learned that N^{α}-(β-Asp)Lys was present in many samples, which is in agreement with the findings of Lou [9].

Discussion

In his original abstract on N^{ϵ}-(β-aspartyl)lysinuria Frimpter supposed a connection of this peptide with a new syndrome. The symptoms of his patient, including growth failure and intractable vomiting, favoured this hypothesis. However, both the gradual disappearance of the isopeptide in his patient and our finding of this compound in children with various pathological conditions are contradictory to the existence of a common defect.

A genetic basis for N^{ϵ}-(β-aspartyl)lysinuria does not seem likely because of its transient character in Frimpter's patient and our patient I.D. Moreover our patient D.L. had a sister B.L. with identical symptoms but without N^{ϵ}-(β-aspartyl)lysinuria.

Although Frimpter considered N^{ϵ}-(β-Asp)Lys to be a unique peptide, other β-aspartyl peptides are known to occur in normal human urine, e.g. (β-Asp)Gly and (β-Asp)Ser [10], (β-Asp)His [11].

Although we have quantified N^{ϵ}-(β-Asp)Lys only in those patients having the highest excretions, we have the impression that a ninhydrin-positive compound in this position of the two-dimensional chromatogram occurs rather frequently in small amounts.

Many speculations can be made about the origin of this peculiar dipeptide. The possibility of endogenous formation from lysine and aspartate has to be considered. We could not find any report suggesting such a formation. Another possibility is the existence of a β-aspartyl transpeptidase, in analogy to γ-glutamyl transpeptidase, which catalyzes the transfer of aspartate to the ε-amino group of lysine.

A related peptide, N^{ϵ}-(γ-Glu)Lys has recently been found to act as a cross-link in human proteins [12,13], especially in epidermis, fibrin and hair. N^{ϵ}-

(β-Asp)Lys may have a similar function. In that case we have to assume that the dipeptide is the residue which remains unhydrolyzed after degradation of the corresponding protein fragment. Then we have to explain why this residue is hydrolyzed in some patients and not in others. A possibility is exaggerated degradation of crosslink-containing proteins. N^{ϵ}-(β-Asp)Lys and N^{ϵ}-(γ-Glu)Lys were both detected in cow colostrum [14] after treatment with a mixture of proteolytic enzymes, indicating the presence of N^{ϵ}-(β-Asp)Lys and N^{ϵ}-(γ-Glu)-Lys crosslinks. We did not investigate the presence of N^{ϵ}-(γ-Glu)Lys in these patients. Theoretically the isopeptide may arise from dietary protein. The higher excretion of related peptides like N^{α}-(β-Asp)Lys and N^{α}-(γ-Glu)Orn during the non-fasting state as compared with fasting periods could point to an exogenous origin [9]. Neither evidence for a bacterial formation of the isopeptide in the gut, nor evidence against such formation, was present. Finally isomerisation in vivo or in vitro of N^{ϵ}-(α-Asp)Lys to N^{ϵ}-(β-Asp)Lys has to be considered [15]. At this stage of investigation we still do not know the exact origin of the isopeptide nor whether its excretion can be related to disease. However, it is necessary to be aware of its presence in human urine and to know its position in the chromatographic systems for amino acid analysis.

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