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THE ISOLATION AND CHARACTERIZATION OF AN INSULIN-RELEASING TETRAPEPTIDE FROM URINES OF PATIENTS WITH LIPOATROPHIC DIABETES

K.L. Reichelt, J.H. Johansen*, K. Titlestad* and P.D. Edminson

The Pediatric Research Institute, The University Hospital, Rikshospitalet, Oslo 1, Norway

*Department of Chemistry, University of Oslo, Blindern, Oslo 3, Norway

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SUMMARY: The tetrapeptide pyroGlu-Glu-Asp-GlyOH and its γ-amide have been isolated from the urines of lipodystrophic patients with insulin-resistant diabetes. Both peptides induce insulin release only at high blood glucose levels.

Congenital generalized lipodystrophy is characterized by: a) generalized absence of fat depots, b) increased growth rate with acromegaloid features and advanced bone age, c) a hypermetabolic rate without hyperthyroidism, d) abnormal skin pigmentations such as acanthosis nigricans, and e) the development of an insulin-resistant diabetes after puberty with hyperinsulinaemia and hypertriglyceridaemia (1). Abnormal patterns of urinary glycoprotein-peptide-benzoic acid complexes were obtained on G-25 gel filtration (2). The peptide, isolated and characterized from the patients' urines, had identical properties to the synthetic peptide pyroGlu-Glu-Asp-GlyOH. A second isolated peptide gave identical Rf values (cochromatography) to the γ-amidated synthetic peptide pyroGlu-GLN-Asp-GlyOH. All the peptides stimulated the release of insulin when blood glucose levels were elevated.

METHODS

Urines from lipodystrophic patients were collected on a 24 hour basis, and either frozen or kept cold with 0.5 g thymol/liter urine added. After thawing, the urines were immediately treated by:

a) Urine precipitation with benzoic acid as first described by Chalmers (3). The pH of the urine is adjusted to 4.3 with HCl and 10% by volume of benzoic acid saturated-ethanol added with stirring. As the pH after this precipitation varies considerably, the pH is again adjusted to between 4.0 and 4.3. The urines are left to precipitate overnight at 4°C.

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b) Washing of the benzoic acid precipitate was started by carefully decanting the supernatant and centrifuging the precipitate at 3000 g for 20 min at 4°C. The precipitate was repeatedly suspended in ethanol by stirring and centrifuged, until the supernatant had an absorbance as close to 0.3 as possible at 280 nm with a 1 cm light path. Further washing will cause the glycoproteins to dissolve with the complexed benzoic acid. Some glycoproteins and acidic proteins are very soluble in acids such as perchloric acid or trichloracetic acid (4,5). The washing procedure is critical for reproducibility.

c) G-25 Sephadex (superfine) gel filtration was performed at 4°C using columns, 2.5 x 160 cm, and 0.1 M NH_4HCO_3 buffer pH 8.5. The application volume was 40 ml of buffer per 24 hour urine and the rate of elution was 30 ml/h. Continuous UV_{280nm} recording of the effluent was performed using an ISCO UA5 monitor. Fractions of 15 ml were collected.

d) Release of peptides from glycoproteins and separation from benzoic acid. Glycoproteins, albumin and mucopolysaccharides will bind aminoacids and peptides that can be released by acid (6,7). The second major peak (see ref.2) from the G-25 column (600 - 900 ml) was lyophilized and treated with acetic acid to a final concentration of 0.5-1 M. 8 ml of supernatant were applied onto a Biorad P2 gel column (1.6 x 90 cm) and eluted with 0.5 M acetic acid. The rate of elution was 3 ml/10 min and 3 ml fractions were collected. 10 percent aliquots of each fraction were analyzed for ninhydrin colourable material before, and after, hydrolysis (see detection methods). Continuous monitoring at 254 and 280nm was performed with an ISCO UA5 detector.

e) Peptide purification. An outline is shown in Fig. 1. The following columns were used:

1. Fractogel MG 2000 (Merck) (1.6 x 70 cm in 0.2 M formic acid). Application volumes were 5 ml and rate of elution 3 ml/10 min, and 3 ml fractions were collected. This gel shows very high resolution and retards acidic peptides and aromatic groups. Amidated peptides are eluted as if the apparent MW was larger than the deamidated peptide.
2. Ion exchange.
   Dowex I x 4 (100-200 mesh) was used in the formate form. Column dimensions were 1 x 20 cm and it was eluted step-

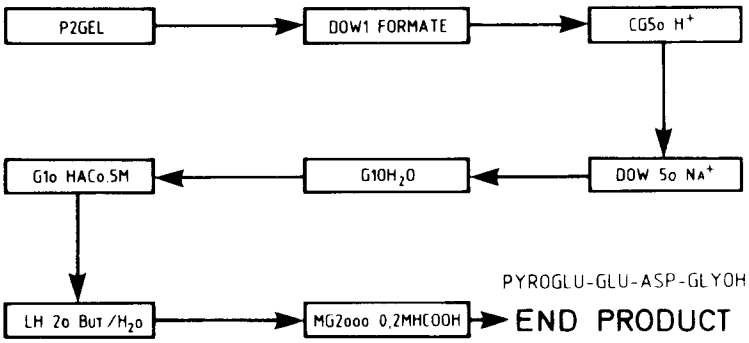

Fig. 1. The overall purification scheme for the factor is shown, and in the sequence used. The final products pyroGlu-Glu-Asp-GlyOH and pyroGlu-Gln-Asp-GlyOH were separated on a Fractogel column in 0.2M formic acid (fig. 3).

wise with 2.5 ml fractions using 24 ml H_2O, 24 ml 0.2 M HCOOH and 30 ml 4 M HCOOH.
Dowex 50 or Aminex A-5 was used with the Na-citrate buffer, pH 2.2, used for amino acid analysis on the Biochrome 2000 analyser, using 0.8 x 80 cm columns. CG 50, a weak cation exchanger was used in the H^+ form in 1 x 20 cm columns. The application volume was 5 ml H_2O and the column was eluted stepwise, in 2.5 ml fractions, with 24 ml 2 M formic acid and 50 ml 2 M pyridine.
The N-substituted peptides pass through the Aminex A-5 cation exchanger at pH 2.2. Most free alpha-amino peptides and amino acids are retained.

3. Sephadex G-10 in water was used to take advantage of the retardation of aromatic compounds at low ionic strength. Column dimensions: 1.6 x 24 cm. Application volume: 3 ml. Rate of elution: 3 ml/10 min with deionized and distilled water.
4. Sephadex LH 20 (Pharmacia) was used in two modes:
   a) n-butanol/water (100/6 by volume). Column size: 2.6 x 24 cm. Application volume: 3 ml. Rate of elution: 18 ml/h.
   b) Methanol/water/0.5 M acetic acid (90/10/0.2). Column size: 2.6 x 30 cm. Application volume: 5 ml. Rate of elution: 18 ml/h.
5. Sephadex G-10 in 0.5 M acetic acid (0.6 x 90 cm) was also used. 3 ml fractions were collected at an elution rate of 3 ml/10 min. The application volume was 5 ml.

f) Detection methods. 10% aliquots of each column fraction were dried overnight in an oven. The material was then hydrolyzed in 2 M KOH, neutralized and the ninhydrin colour developed as described (8). The method has close to equimolecular absorption coefficients for amino acids at 570 nm. By comparing hydrolyzed to unhydrolyzed data, a rough idea of the peptide length could be obtained.

g) Structural studies. Overall amino acid composition was determined by acid hydrolysis for 24 hours in 6 M HCl. The HCl was removed over KOH in a vacuum and the amino acids analyzed on a Biochrome 2000. The peptide did not show any absorbtion at 280nm. N-terminal analysis was performed after methanolysis in 6 M HCl in anhydrous methanol (9) followed by dansylation (10), hydrolysis of the dansylated peptide, and TLC on micropolyamide (11). Pyroglutamic acid was also removed with pyroglutamyl peptidase (gift from Dr. Lill, Boehringer A/G, Mannheim, Germany) and isolated by anion exchange chromatography on Dowex 1 (8). Stepwise Edman degradation was carried out with dansylation as described by Gray (12).
To test for C-terminal amidation, complete hydrazinolysis was performed (13), but dansylated amino acids were analyzed for rather than the dinitrophenyl-amino acids used by Fraenkel-Conrat (13).

h) Criteria of purity of the isolated peptide were based on the following: 1) Constant and integral amino acid composition in spite of further purification steps. 2) Same composition at the beginning, middle and terminal part of the final peptide peak. 3) The presence of only one N-terminal amino acid. In the case of the N-substituted peptides this may be difficult as some hydrolysis of especially the aspartyl-peptide bonds may occur during methanolysis. Only one C-terminal amino acid should be present.

i) Synthesis was performed by conventional techniques (Johansen et al, in prep.). The synthetic peptide and analogues have been tested by cochromatography and bioassay.

Bioassay:

Animals: Male Sprague-Dawley rats weighing 230-300 g, were supplied by Alab AB, Sollentuna, Stockholm. A catheter was inserted into vena jugularis under Athesin^{R} anaesthesia. After the surgical procedure, the rats were allowed to recover for 12 hours. Experiments were performed after an overnight fast; food was withheld during the experiment.

Experimental Procedure

Peptide and glucose in physiological saline were administered intravenously to overnight fasted rats via the catheter. The blood samples were drawn before, and at 2, 5, 10 and 30 min after peptide/glucose administration. Heparinized 50 ul capillary tubes were used to obtain blood which was analysed for glucose by a glucose oxidase method. Plasma was rapidly separated and, prior to freezing, a volume of Trasylol (10.000 KIU/ml) was added, corresponding to 10% of the plasma volume. Insulin was measured by radioimmunoassay (14).

Results are expressed as the mean ± S.E.M. The data were analysed by standard statistical methods using Student's t-test. A p value of less than 0.05 was considered significant.

RESULTS

The G-25 profile has been published elsewhere (2). The elution profile of the acid-dissociated fractions on a P2 gel are shown in figure 2, where the activity studied was found mainly in the peak labelled II. The overall purification scheme is shown in figure 1. Some relevant chromatographic data are illustrated in figure 3, with the bio-active areas marked with arrows. We primarily purified the second peak in fig. 3, but peak I had the same amino acids on hydrolysis and the same bio-activity.

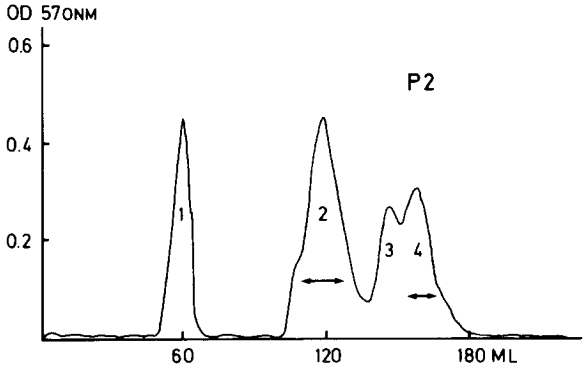

Fig. 2. Elution profile on a P2 gel column in 0.5 M acetic acid. Detection with ninhydrin after hydrolysis of aliquots of each fraction. Stimulation of insulin release was found in two different fractions. The compound isolated is found in peak II.

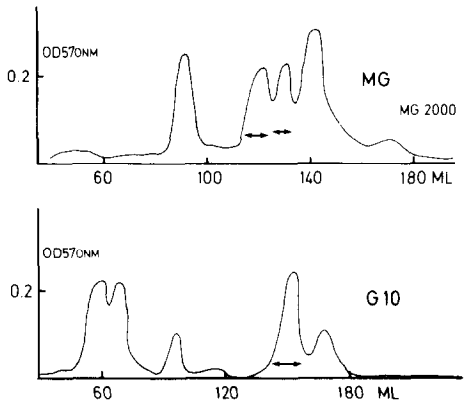

Fig. 3. Elution profiles on Sephadex G-10 in 0.5 M acetic acid, and Fractogel MG 2000 in 0.2 M formic acid. Detection as in fig. 2. The arrows indicate the two active peptides finally isolated. Peak II (Fractogel MG 2000) contains pyroGlu-Glu-Asp-GlyOH, while peak I cochromatographs with synthetic pyroGlu-Gln-Asp-GlyOH.

The peptide which released insulin only in the presence of high blood glucose has the following properties: It passes through the anion exchanger, in the formate form, in the aqueous eluate and must therefore have a neutral or basic C-terminal group. It passes through both Dowex 50, at pH 2, and CG 50, in the H^+ form, in the aqueous eluate and, as it is not anionic, must be N-substituted or cyclic. Only the extremely anionic amino acids taurine and cysteic acid pass through Dowex 50 at pH 2. As the peptide is not excessively retained on Sephadex G-10 in water and has no absorbtion at 254 and 280nm, it cannot contain any aromatic groups. It is not retained on LH-20 columns and does not, therefore, contain hydrophobic regions such as found in leukotrienes. The peptide has a low molecular weight as shown by gel filtration in 1M acetic acid on Sephadex G-10, or in 1.0M formic acid/40 mM HCl on Fractogel MG 2000, where it elutes after the void volume.

Total amino acid analysis gave Glu^2,Asp^1,Gly^1. (The average of three hydrolyses: Glu 2.3, Asp 0.93, Gly 1.35). The peptide did not give any ninhydrin colour without hydrolysis, confirming its N-substituted nature. The first eluting compound (fig. 3, peak I) contained Glu 1.9, Asp 1.1 and Gly 0.9. Hydrazinolysis indicated non-amidated-glycine as the C-terminal, determined as dansyl-glycine. After methanolysis, dansyl-Glu was obtained and traces of dansyl-Asp. The latter was probably due to splitting of the labile aspartyl-peptide bond in 6 M HCl in methanol. The second

dansylation step after one Edman cycle yielded dansyl-Glu as well. The only reasonable structure was, therefore, pyroGlu-Glu-Asp-GlyOH. As we could not determine the amide content with any certainty, the analogues pyroGlu-GLN-Asp-GlyOH and pyroGlu-Glu-ASN-GlyOH were synthesized (Johansen, to be published). Bioactivity was found for only pyroGlu-GLN-Asp-GlyOH. The peptide pyroGlu-Glu-Asp-GlyOH cochromatographed with the urine-derived peptide isolated from peak II, figure 3, thus confirming the structure. The analogue pyroGlu-GLN-Asp-GlyOH cochromatographed with the activity in peak I, figure 3. Deamidation occurs readily in vivo and in vitro, and the endogenous peptide may therefore be amidated. No trace of carbohydrate was found.

Intravenous injection of 4.36 mg/kg of the peptide alone, in physiological saline, into fasted animals was without effect on blood glucose and insulin levels. With increasing levels of blood glucose, obtained by infusion of 640 mg/kg or 1300 mg/kg glucose, significant additional increases in insulin release were found at 2, 5 and 10 minutes ($p<0.05$) as shown in Table 1.

Blood glucose levels were significantly lower with the two highest glucose infusions when the peptide was present. With 1300 mg/kg glucose, 20.6 ± 0.6 (x ± sd) mmoles of glucose/liter blood were found compared with 19.6 ± 0.6 mmoles/l in the presence of peptide (at 5 minutes $p < 0.01$, n=8).

The effect of peptide (4,36 mg/kg) on insulin release was the same as with 20 mg/kg tolbutamide (Edman:personal communication). Due to the low levels of peptide material in normal urines, we

Table 1. Effect of 4.36 mg/kg of peptide on insulin release in rats (n=4 for each group)

Glucose mg/kg	Peptide	0'	2'	5'	10'	30'
				uU/ml		
80	-	22 ± 3	39 ± 6	21 ± 3	19 ± 3	22 ± 3
80	+	15 ± 3	38 ± 6	22 ± 4	20 ± 4	19 ± 2
320	-	8 ± 3	50 ± 7	31 ± 6	28 ± 2	15 ± 3
320	+	12 ± 2	56 ± 4	41 ± 4	31 ± 3	14 ± 3
640	-	9 ± 3	58 ± 7	67 ± 2	40 ± 2	9 ± 2
640	+	12 ± 2	92 ± 8	88 ± 9	60 ± 6	10 ± 2
1300	-	15 ± 3	72 ± 6	80 ± 6	74 ± 5	24 ± 4
1300	+	21 ± 3	118 ± 13	124 ± 8	111 ± 7	25 ± 3

At 2 min., the peptide pyroGlu-Glu-Asp-GlyOH gave significantly higher insulin levels with 640 mg/kg glucose infused ($p < 0.05$). At 5 min., $p < 0.05$ and at 10 min. $p < 0.01$. For the highest glucose concentration $p < 0.01$ at 2, 5 and 10 min.

cannot determine if the peptide is present without immunological assay techniques.

DISCUSSION

Metabolically active peptides have been found in urine in several diseased states. A capillary endothelial cell migration factor has been found in patients with transitional-cell carcinoma (15); fat mobilizing activity of human urine has been described (3); and we have isolated an urinary tripeptide which blocks the cephalic phase of digestion (16,17,18,19) although not anorexogenic as first reported. Growth hormone-release-inhibitory activity has been found (20) and a sleep-inducing muramyl peptide (21) occurs in urine, as does LHRH-like activity (22). In schizophrenia, a whole family of peptide factors, that are increased over normal levels, have been found (23,24,). These have neurochemical effects (23) and the data have been confirmed for serum (25). It is, therefore, not surprising that we find an insulin-releasing factor in urines of insulin-resistant diabetics typical of postpubertal generalized lipodystrophy (1). Acid soluble peptides in urine have been found to be relatively resistant to proteinase and peptidase breakdown (26) and reflect endogenous metabolic states more than nutritional inputs (26). We do not know where the peptide is formed or released. This must be the object of future studies. The peptide should be of interest in treatment of type II (ageing) diabetes.

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