The Amino Acid Composition of a Melanophore-expanding Peptide from Pig Pituitary Gland

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The amino acid composition of a melanophore-expanding peptide isolated from pig pituitaries has been investigated by chromatography on Dowex 50 columns. Tryptophan was determined spectrophotometrically. The analyses indicate the following 18 amino acid residues in the molecule (mol. wt. 2176):

Asp, Glu, Gly, Ser Met Pro, Phe Tyr Try His Lys, Arg.

In a previous communication ¹ the isolation from acetone-dried pig posterior lobe powder, of an electrophoretically homogeneous fraction, a peptide, possessing a high melanophore-expanding activity, has been reported. Isolation and amino acid analysis of a melanophore-expanding hormone from pig pituitary gland have also been reported by Lerner and Lee ², and by Benfey and Purvis ³. Their substances differed considerably in the amino acid composition, and this disagreement prompted the publication of the present investigation.

In the following experiments acid hydrolysates of the peptide have been analyzed by chromatography on columns of Dowex 50—X4 and of Dowex 50—X8, using the methods of Moore and Stein ^{4,5}. The concentrations of the amino acids in the effluent fractions were in all experiments determined by the modified reagent described by Moore and Stein ⁶. Tyrosine and tryptophan have been determined spectrophotometrically according to Goodwin and Morton ⁷, and the occurrence of cystine and cysteine have been tested by paper electrophoresis of a sample previously oxidized with performic acid according to Thompson ⁸ and then hydrolyzed with 6 N hydrochloric acid.

The investigation was started with chromatography on columns of Dowex 50—X4. This method, however, did not give satisfying separations of all the present acids, and, furthermore, difficulties arose in the determination of the correct base-line (caused by ammonia) in the vicinity of the lysine and histidine peaks. Complementary analyses were therefore performed according to the older method of Moore and Stein on Dowex 50 of the stronger crossed-linked type. All the methods mentioned above have given a satisfactory determination of the amino acid composition.

EXPERIMENTAL

From a pure specimen, dried over phosphorus pentoxide *in vacuo*, samples weighing about 2 mg were taken for analysis. The peptide was dissolved in 1.0 ml 6 N hydrochloric acid and hydrolyzed in a sealed Pyrex tube. The hydrolytic conditions were varied (see Table 1) in order to make sure that all peptide linkages were split and in order to obtain indications of the reaction orders of the amino acids being decomposed. The hydrolysate was quantitatively transferred to a small beaker (5 ml) and the hydrochloric acid removed over sodium hydroxide pellets in a vacuum desiccator. The dry residue was dissolved in 1 ml buffer solution, according to the directions given by Moore and Stein. When using Dowex 50-X4, the column was 0.9×150 cm; for the separations on Dowex 50-X8, the column used for the acidic and neutral acids was 0.9×115 cm; and that used for the basic amino acids was 0.9×15 cm. The fractions were collected by means of an automatic fraction collector and analyzed for the amino acids with ninhydrin. The optical density readings were made with a Beckman B spectrophotometer. The integration for each amino acid was performed by simple addition of the ninhydrin color values (in leucine equivalents in micromoles), after subtracting the base-line value near the amino acid peak. With the modified ninhydrin reagent an investigation of the color yields of the amino acids was carried out. The acids were dissolved in the buffer solutions, in which they emerge from columns of Dowex 50-X4 and of Dowex 50-X8 in the current chromatographic procedures. The found values agree, within the experimental errors, with those which have been given by Moore and Stein⁴ when using the buffers described by them for Dowex 50-X4.

The substances in the different peaks of the column chromatograms were identified by paper chromatography, paper electrophoresis, or specific reactions, according to the following procedure. In order to locate the different peaks, 0.5 ml of every other fraction (1 ml) was boiled with ninhydrin. All fractions which belonged to the same peak were then desalted on ion exchange resin according to Drèze, Moore and Bigwood⁵, using columns of 0.9×10 cm. The salt-free eluates were then evaporated to dryness. For the identification of the acidic and neutral amino acids paper chromatography was used, and for that of the basic amino acids paper electrophoresis was used. In addition, the Pauli test, modified according to Sanger and Tuppy¹⁰, and the reaction of Sakaguchi, as described by Jepson and Smith¹¹, gave an additional identification method for histidine and arginine respectively.

The presence of methionine could only be demonstrated in one of the methods of Moore and Stein^{4,5}, and therefore it was necessary to confirm the presence of this amino acid by an independent method. One sample of the intact hormone and one of methionine were oxidized with performic acid according to Thompson⁶. The oxidized hormone, free from hydrogen peroxide, was hydrolyzed for 30 hours at 120° with 6 N hydrochloric acid. A first separation of the acids was achieved by paper electrophoresis in 0.05 M borate buffer (pH = 8.7), when using an apparatus constructed by Porath and Jerstedt¹², which permits electrophoresis in one direction and succeeding chromatography in the perpendicular direction. After the electrophoresis the paper was dried, the oxidized methionine was put on near the edge of the paper as a reference, and the acids were separated by descending chromatography in a system of eighty parts of pyridine and twenty parts of the 0.05 M borate buffer.

An independent investigation of the occurrence of cysteine and cystine was also made. One sample of the peptide and one of cystine were simultaneously oxidized with performic acid according to Thompson. The peptide hydrolysate was subjected to paper electrophoresis at pH 4.9, using 0.1 M pyridine-acetic acid buffer. The oxidized cystine and a mixture of cysteic acid, aspartic acid, glutamic acid, and lysine were used as references.

For the analysis for tyrosine and tryptophan⁷, 1.5 mg of the peptide was dissolved in 5 ml 0.1 M NaOH. Readings of the ultraviolet absorption at 280 and 294.5 $m\mu$ were made with a Beckman DU spectrophotometer using 1 cm quartz cells. The molar extinction coefficients for tyrosine and tryptophan were determined at the same wave-lengths, and from the measurements it was possible to calculate the molar ratio of tyrosine to tryptophan.

In order to find out if the ammonia in the total hydrolysate originated from amide nitrogen or from another source, the amide nitrogen of the peptide was determined by the Conway microdiffusion technique. The Conway vessels were provided with a liquid seal according to Öbrink¹³, and the procedure was essentially the same as that of Hirs, Stein and Moore¹⁴. 3.28 mg of the peptide was dissolved in 1.00 ml of 1 N H₂SO₄. The solution was divided into two equal parts and heated in sealed Pyrex tubes at 100°, one tube for 4 hours, the other for 8 hours. 0.2 ml aliquots were transferred from the tubes to the Conway vessels containing 0.4 ml of 0.02 N H₂SO₄ in the inner chamber. The sample in the outer chamber was brought to pH 10 to 11 by addition of 1 ml of the alkaline solution (see Hirs *et al.*¹⁴), which was also used in the seal of liquid. After standing overnight at room temperature, 0.15 ml aliquots from the inner chamber were analyzed for NH₃ with the modified ninhydrin reagent⁶. The technique was checked on an electrophoretically homogeneous preparation of asparagine.

RESULTS AND DISCUSSION

The elution curve obtained from a run on a column of Dowex 50—X4 is presented in Fig. 1. The time of hydrolysis was 30 hours at 120°. The separation of the acids was not as good as was expected. This was especially the case for tyrosine and phenylalanine, which emerged together as one symmetric peak. There was no separate ammonia peak, but ammonia caused an elevated base-line color in the region of lysine and histidine, which made the exact determination of these acids difficult. The ninhydrin-positive peak X, only given by this method and not by the Dowex 50—X8 method, could not be identified as one of the common amino acids.

A two-dimensional chromatogram run with a hydrolysate of the peptide prepared under identical conditions to those mentioned above gave no spot which could account for X. The system used was *n*-butanol-acetic acid-water, followed by phenol, pH 9.3 borate buffer¹⁵.

In Figs. 2 and 3 the results obtained, respectively, with a long and short column of Dowex 50—X8 are shown. The time of hydrolysis was 40 hours at 120°. The amino acids were all completely separated except tyrosine and phenylalanine. A peculiarity, however, was that this method did not give any methionine.

The check of methionine as methionine sulfone (see Experimental) gave a distinct spot, which in color and position agreed with the only spot given by the simultaneously oxidized methionine. The occurrence of methionine was further confirmed by the sulphur content and by the absence of cysteine and cystine. The peptide was found to contain 1.47 % S, which corresponds to one gram atom sulphur per mole (molecular weight approximately 2 500, as obtained by a recent determination at this Institute by the method of Archibald¹⁶), and as the test of cysteine and cystine gave a negative result (see Fig. 4), the total sulphur must come from methionine.

In Table 1 the analytical data from various experiments on Dowex 50 columns are collected. From the values in the table it appears that 15 hours at 115° is too short a time for complete hydrolysis, and that a hydrolysis for 50 hours at 120° is rather long. Especially serine is decomposed to a large extent.

The runs on short columns of Dowex 50—X8 gave a separate ammonia peak (Fig. 3). If the ammonia values in the table are corrected for ammonia

Fig. 1. Elution curve for a 30 hour hydrolysate of the peptide at 120° on a 0.9×150 cm column of Dowex 50-X4. For the run shown 1.874 mg of peptide hydrolysate was used.

from the destruction of serine, they indicate the presence of one amide group in the molecule. When using the Conway microdiffusion technique, however, the found ammonia values, after 4, respectively 8 hours of hydrolysis, were 3.8 and 4.4 μ moles per 100 mg of the peptide, which is only about 0.1 amide group per molecule. The check of the method on asparagine gave after 4 hours of hydrolysis 1.0 ± 0.1 amide group per molecule. Thus it seems necessary to assume another source of the ammonia in the total hydrolysate than amide nitrogen.

The molar ratio of tyrosine to tryptophan was found to be $^7 1.07$, and this together with the values in the table give the following empirical formula of the peptide:

Fig. 2. Elution curve for the determination of the acidic and neutral amino acids on a 0.9×115 cm column of Dowex 50-X8. The time of hydrolysis was 40 hours at 120° . For the run illustrated 1.990 mg of peptide hydrolysate was used.

Fig. 3. Elution curve for the determination of the basic amino acids and ammonia on a 0.9×15 cm column of Dowex 50-X8. The time of hydrolysis was 40 hours at 120° . For the run illustrated 1.851 mg of peptide hydrolysate was used. The large peak, A, comprised all the amino acids emerging before tyrosine and phenylalanine.

Fig. 4. Electrophoretic patterns of 1) cystine oxidized according to Thompson, 2) the active peptide oxidized according to Thompson and then hydrolyzed for 20 hours at 120°, 3) a mixture of cysteic acid, aspartic acid, glutamic acid, and lysine.

The results in this analysis differ considerably from those given by Lerner and Lee ², who reported the presence of alanine, valine, cystine, leucine and threonine, which were not found in this investigation. Furthermore, they did not find histidine and did not analyze for methionine. Benfey and Purvis ³ reported the same amino acids as found in this work, but their minimum number of residues per molecule were twice those in Table 1 for all acids except glycine, methionine and proline.

Table 1. Analytical data from various experiments on Dowex 50 columns.

Ion exchange	Dowex 50—X4			Dowex 50—X8				Calculated integral numbers of residues for mol. wt. 2 500 *
Dimension of the column	0.9 × 150 cm			0.9 × 115 cm		0.9 × 15 cm		
Hydrolytic conditions	15 h at 115°	30 h at 120°	50 h at 120°	40 h at 120°	40 h at 120°	30 h at 120°	40 h at 120°	
Amount of hydrolysate	1.974 mg	1.874 mg	1.865 mg	1.832 mg	1.990 mg	1.832 mg	1.851 mg	
Amino acids	Micromoles/100 mg peptide							
Aspartic acid	83.6	90.6	81.0	84.3	84.6			2
Glutamic acid	75.4	83.9	86.1	88.2	86.6			2
Glycine	84.9	85.1	84.5	86.2	85.4			2
Serine	38.9	36.7	20.2	22.3	28.9			1
Methionine	34.5	42.3	36.5					1
Proline	126.5	129.6	124.0	113.7	133.6			3
Phenylalanine	29.3	37.0			43.2	} 73.5	} 79.1	1
Tyrosine	30.3	37.0			36.3			1
Histidine	42.1	43.6	46.8			41.8	42.0	1
Lysine	77.2	90.0	88.7			85.9	91.2	2
Arginine	41.5	41.4	42.7			38.8	43.3	1
Ammonia						53.2	69.0	

* The approximative value found in the ultracentrifuge. The molecular weight calculated on the basis of the integral numbers of residues including the tryptophan residue, amounts to 2 176.

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Added in proof: Since this manuscript was submitted for publication the amino acid analysis has been repeated on a Dowex 50—X4 column. The results obtained were in complete agreement with those reported in the text, except that in this case the unidentified component X did not appear. The author is indebted to Mr. M. Rees, Department of Biochemistry, Cambridge, for his assistance in carrying out this analysis.]

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