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ISOELECTRIC POINT DETERMINATION OF HUMAN AND CAMEL β-ENDORPHIN , α-ENDORPHIN AND ENKEPHALINS.

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SUMMARY: The isoelectric point of the camel and the human β-endorphin, of the α-endorphin and the enkephalins were determined by analytical isoelectric focusing on 1 mm thin polyacrylamide gel slab. The difficulty of staining peptides as short as β-endorphin or smaller was overcomed using a modification of Bibring and Baxandall's or Faupel and Von Arx's staining method. The camel β-endorphin gives two bands having isoelectric point of 10.3 and 10.4, the human β-endorphin focus at pH 9.9, while α-endorphin, leu and met-enkephalin at pH 5.9, 5.5 and 5.45 respectively. The staining method described coupled with the isoelectric focusing seems to be fit for discriminating β-endorphin in a crude rat pituitary extract.

Since the discovery of β-endorphin at first in pituitary gland of mammals (1-5) and then in various tissues (6-8), several authors carried out its isolation and purification with different techniques (3,8-11) and studied its structure-function relationship (11-12). Although the purification methods, particulary through high pressure liquid chromatography coupled with radioimmunoassay determination are satisfactory and well standardized, the chemical parameters of this polypeptide are not yet completely defined.

In order to improve the knowledge of such parameters, we determined the isoelectric point (pI) of this substance and of some related polypeptides using isoelectrofocusing (IEF) on polyacrylamide thin gel slab and special staining procedures.

MATERIALS AND METHODS

Acrylamide; N-N'methylenbisacrylamide (Bis); ammonium persulphate; tetrametyl ethylendiammine (TEMED); Amberlite monobed resin MB-1; trichloroacetic acid (TCA); L-histidine; glycerol; N-2-hydroxyethylpiperazine, N'-2-ethanesulphonic acid (HEPES) from BDH Chemicals LTD Poole England were used.

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Coomassie brilliant blue R-250; NaOH; HCl; glutamic acid from Merck Darmstadt. Pharmalyte and Silane A-174 were purchased from Pharmacia-fine chemicals. Human β-endorphin (β-lipotropin 61-91); camel β-endorphin (β-lipotropin 61-91) and α-endorphin (β-lipotropin 61-76) from Peninsula Laboratories; leu--enkephalin and met-enkephalin from Miles Yeda L.T.D..

Analytical electrofocusing on thin layer polyacrylamide gel was performed as follows.

IEF technique: Instead of using the usual commercial thick slab (2mm) a thinner (1mm) home-made gel slab was used (size 11x11 cm). The gel contained 7% acrylamide (the ratio of acrylamide to Bis being 25:1) and 30% glycerol. For the pH range 8.0-10.5 or 4.0-6.5, 6.33ml of appropriate Pharmalyte pH interval to 100ml of acrylamide monomers were added. When the pH range was 6.0--10.5, 100ml of acrylamide monomers contained a cocktail of Pharmalyte pH interval 4.0-6.5, 6.5-9.0 and 8.0-10.5 in the ratio respectively of 1.67, 1.67 and 3 ml.

The gel support was a glass plate soaked into a solution of Silane (8 ml dissolved in 2 l of distilled H_2O, aggiusted to pH 3.5 with acetic acid) then dried with air. To polymerize the gel, 200 µl of ammonium persulphate solution (22.8 mg/ml) and 30 µl of TEMED were added.

With the pH range of 8.0-10.5 1M NaOH was the catholyte and 0.25M HEPES the anolyte; 0.04M glutamic acid was used as anolyte when the pH range was 6.0-10.5; 0.1M L-histidine and 0.1M glutamic acid in 0.5M H_3PO_4 were used as catholyte and anolyte respectively with pH range of 4.0-6.5.

After prefocusing for 30' at 4°C, constant 10W and 1300V max. or costant 20W and 1700V max. when the pH range was 4.0-6.5, the samples dissolved in distilled water (1.5 µg/µl) were applied at the anodic side as droplets or soaked on filter paper strips (up to 10 µl).

The running time, as the same conditions of for the prefocusing, was 3 h.

The pH gradient was then directly measured on the gel by a surface glass pH electrode (LKB 2117-111).

The fixing and staining technique for β-endorphin was a modification of Bibring and Baxandall's method (13): the gel was fixed with gentle agitation in 12% TCA, over 24 h; staining was to equilibrium in 0.002% Coomassie brilliant blue R 250 in 12% TCA over 24 h; then the gel was washed by a solution containing 25 ml glycerol, 80 ml TCA 12% and 105 ml distilled water, in order to eliminate the excess of stain.

To detect peptides shorter than β-endorphin, the method of Faupel and Von Arx (based on Pauly's stain method to detect histidine and or tyrosine containing peptides) was used.

Detection of rat pituitary β-endorphin: 36 female Sprague-Dawley rats weighting 200 g were decapitated. Pituitary glands were quickly removed,pooled and placed in 2 ml of cold mixture containing acetic acid 90% and HCl 0.2 N 10%, then homogenized with Polytron. 0.32 ml of acetone were added. After preheating at 70°C for 45 min, the extraction was processed according to Teschemacher's method (2). The resulting crude pituitary material was desalted on Sephadex G10, lyophilized and then used in IEF experiment.

While the rat β-endorphin is identical to that of camel, the content of β--endorphin like material in this crude extract was previously verified by radioimmunological assay for camel β-endorphin, according to Ogawa's technique (15).

RESULTS

Fig. 1 shows the focusing pattern in pH range 8.0-10.5 of human β-endorphin and camel β-endorphin as stained by Bibring and Baxandall's modified method. It can be seen that these peptides migrate at very high pH and that human β --endorphin is focused as a single sharp band with pI near 9.9, while camel β--endorphin gives two bands, having pI values of 10.3 and 10.4 respectively.

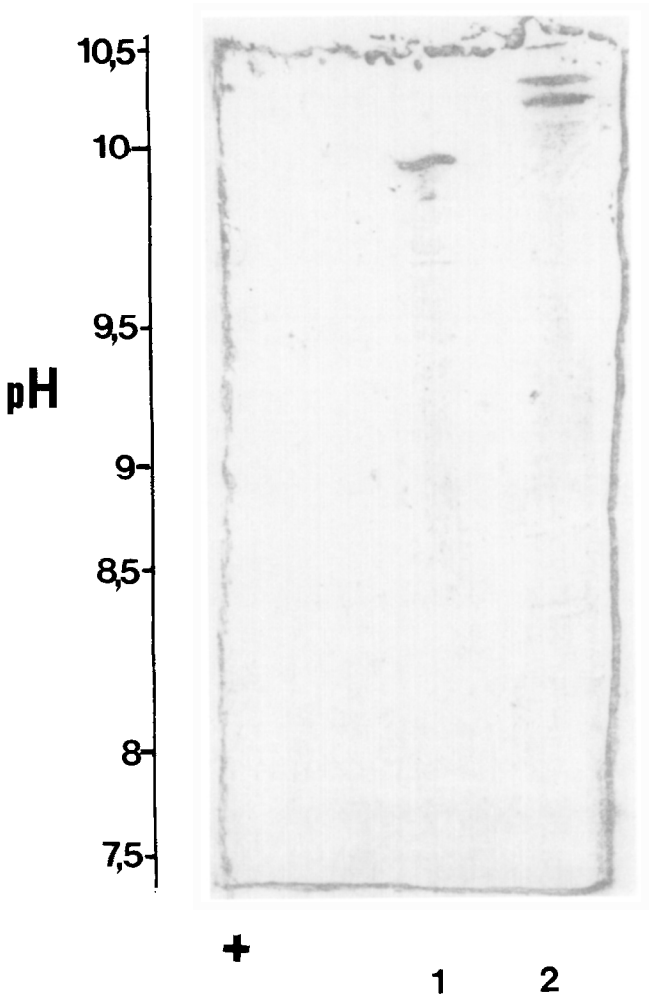

Fig. 1. Electrofocusing pattern in pH range 8.0-10.5 of human β-endorphin (1) and camel β-endorphin (2) stained with Bibring and Baxandall's modified method.

In order to determine the pI value of peptides shorter than β-endorphin we stained the gel (pH range 4.0-6.5) by the Faupel and Von Arx's method and used more quantity of substances: met and leu-enkephalin 200 µg and α-endorphin 125 µg. The pI of leu-enkephalin, met-enkephalin and α-endorphin were respectively 5.45, 5.50 and 5.90 (Table 1).

Finally we also tried to detect by Bibring and Baxandall's modified staining method the rat β-endorphin in the crude pituitary extract. Since in the rat pituitary extract a lot of peptides related to β-endorphin are present, which have pI lower than 8.0, we prepared a home-made thin gel with a wider

TABLE I. Isoelectric point of α-endorphin and enkephalins on polyacrylamide gel, stained with Faupel and Von Arx method.

Peptides	pI
α -endorphin	5.90
leu-enkephalin	5.50
met-enkephalin	5.45

pH range than that used previously. As expected, the pituitary extract (Fig.2) is resolved in many bands, one of which focuses at the same pI as camel β--endorphin (≃10.3).

DISCUSSION

First of all we pointed out that until now no data of other Authors are available to compare our results about the pI of the opioid oligopeptides.

In theory we expected that the isoelectric point of human and camel β --endorphins, would be near pH 8.2-8.4 as approximately calculated from pK values for the ionizing groups of aminoacids contained in their primary structure (16), on the contrary our experiments show for these polypeptides a much higher pI. Such a difference could probably be ascribed to the β- endorphin secondary and tertiary structures (so far not yet completely clarified). Leu, met-enkephalin and α-endorphin have a pI lower than β-endorphins as expected from their primary structures.

With regard to the two bands developed for camel β-endorphin in our experiments, we can suppose that, unlike the human β-endorphin, there are 50% impurities in the commercial preparation we used.However it remains to be verified if both bands or only one have opioid activity and radioimmunologically react with camel β-endorphin antiserum. As far as the rat pituitary extract is concerned, we can conclude that the IEF on polyacrylamide coupled with the described staining technique seems to be fit for well discriminating β-endorphin from the other related peptides. In this regard it is interesting to point out that ovine β-lipotropin have a pI of 6.33 as showed by C.H. Li (12).

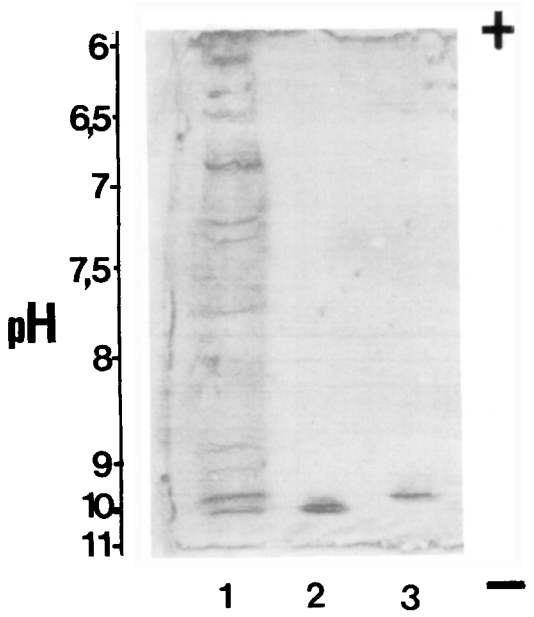

Fig. 2. Electrofocusing pattern in pH range 6.0-10.5 of rat pituitary extract (1), camel β-endorphin (2) and human β-endorphin (3) stained with Bibring and Baxandall's modified method.

Further experiments are now in progress for studying the nature, the radioimmunological reactivity and the opioid activity of all the bands. Some preliminar radioimmunological assays of the substance contained in the rat pituitary extract, focused at the same pI as camel β-endorphin and eluted by the corresponding little piece of slided gel with 0.1 M acetic acid, confirm that this material well reacts with camel β-endorphin antiserum.

Finally it can be concluded that the Bibring and Baxandall's modified method is adequate to stain without loss of resolution bands spaced 0.3 mm and to detect small quantities of oligopeptides (10-15 µg), that with usual Coomassie or specific for oligopeptides staining methods up to now described (17,18) can not be detected because of problems originating from fixation and staining. Even the shorter peptides that can not be stained with Bibring and Baxandall's method can be well identified using the Faupel and Von Arx's, which is less sensitive than that of Bibring and Baxandall, but anyway permits the determination of the pI of these peptides otherwise not detectable. The only trouble in both methods is that after the color development, the contrast is slowly disappearing.

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