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USEFUL MODIFICATION OF PAULY'S REAGENT

DETECTION OF LOW-MOLECULAR-WEIGHT HISTIDINE-CONTAINING PEPTIDES BY ISOELECTRIC FOCUSING

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SUMMARY

An improved method for the detection of low-molecular-weight (<10,000 daltons) histidine-containing peptides on isoelectric focusing is described.

The major advantage of the method is that the staining mixture (a modification of Pauly's reagent) does not react with ampholytes of similar size and pI, permitting accurate determination of the isoelectric points of these peptides.

INTRODUCTION

The high-resolution technique of isoelectric focusing, which utilizes electrophoresis in a pH gradient to separate proteins according to their isoelectric points, is used extensively[1,2]. It has been applied mainly to the separation and characterization of peptides of high molecular weight[2]. Difficulties of detection, peptide loss and the rapid diffusion during conventional staining, as well as the difficulties encountered in distinguishing carrier ampholytes of similar size and pI, have prevented the extension of the technique to the analytical resolution of low-molecular-weight peptides.

We describe here a versatile method for the detection of low-molecular-weight histidine-containing peptides, using a modification of Pauly's[3] staining method.

MATERIAL AND METHODS

The carrier ampholyte solution, pH range 3.5–10, was obtained from LKB (Bromma, Sweden). The calibrated mixture of pH markers, acrylamide and N,N'-methylene bisacrylamide were purchased from Serva (Heidelberg, G.F.R.). Ammonium persulphate was obtained from Fluka (Buchs, Switzerland).

Apparatus

The Flat-sheet Gel Electrophoresis Cell 82100* and the stabilized Power supply 67502 (Camag, Muttenz, Switzerland) were used.

Preparation of the gel

The gel was prepared form the following refrigerated stock solutions: A, 30 g of acrylamide and 1 g of N,N'-methylenebisacrylamide dissolved in 100 ml water; B, 8 *M* urea; C, 1% ammonium persulphate in water.

To prepare the gel, 13.75 ml of stock solution A, 36.50 ml of stock solution B, 3.3 ml of stock solution C and 2.0 ml of LKB ampholines (pH range 3.5–10) were mixed.

Gel polymerization

The mixture was filled into the apparatus by means of a capillary and polymerized within *ca.* 30 min.

Sample application

Samples were easily applied with the aid of small strips of Whatmann No. 3MM filter paper (5 mm × 10 mm). A 50-μg amount of the peptides listed in Table I, dissolved in 10 μl of water, were applied using a 10-μl Hamilton dispenser. The peptides were synthesized in our laboratories.

TABLE I

PEPTIDES USED IN THIS INVESTIGATION

Synthetic peptide	*Spot No.*	*Molecular weight*
p-Glu-His-NH_2	1	265
p-Glu-His-OCH_3	2	280
p-Glu-His-Pro-NH_2HOAc	3	362
H-Asn-Gln-His-OH	4	397
p-Glu-His-Pro-OHHCl	5	409
Val^5-angiotensin II	6	1032
Human calcitonin	7	3318
Porcine ACTH (1–39)	8	4567
Human insulin	9	5808

Electrofocusing conditions

The anodic and cathodic chambers were filled with 10% phosphoric acid and 1 *N* NaOH, respectively. Focusing was performed at a constant temperature of 10°, maintained by circulating cooling water. The voltage was steadily increased in such a way that the current never exceeded 25 mA (Fig. 1). At the beginning of the operation the potential difference between the two electrodes was 80 V, at the end it was 500 V (Fig. 1).

* This horizontal flat-sheet gel electrophoresis cell (System CIBA-GEIGY) was developed in collaboration with Camag.

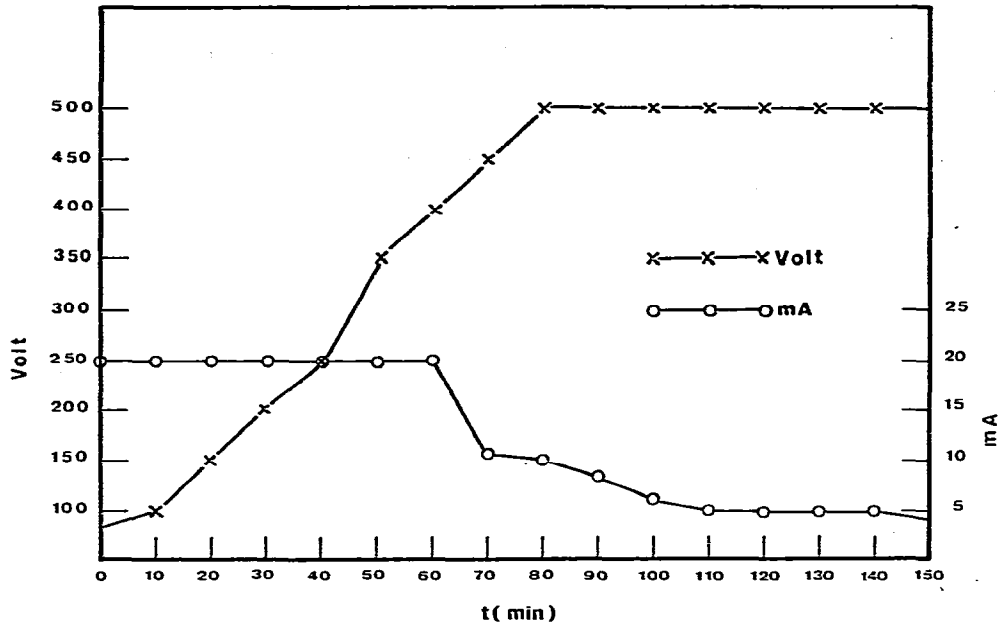

Fig. 1. Record of voltage and current during focusing in polyacrylamide gel (pH range 3.5–10).

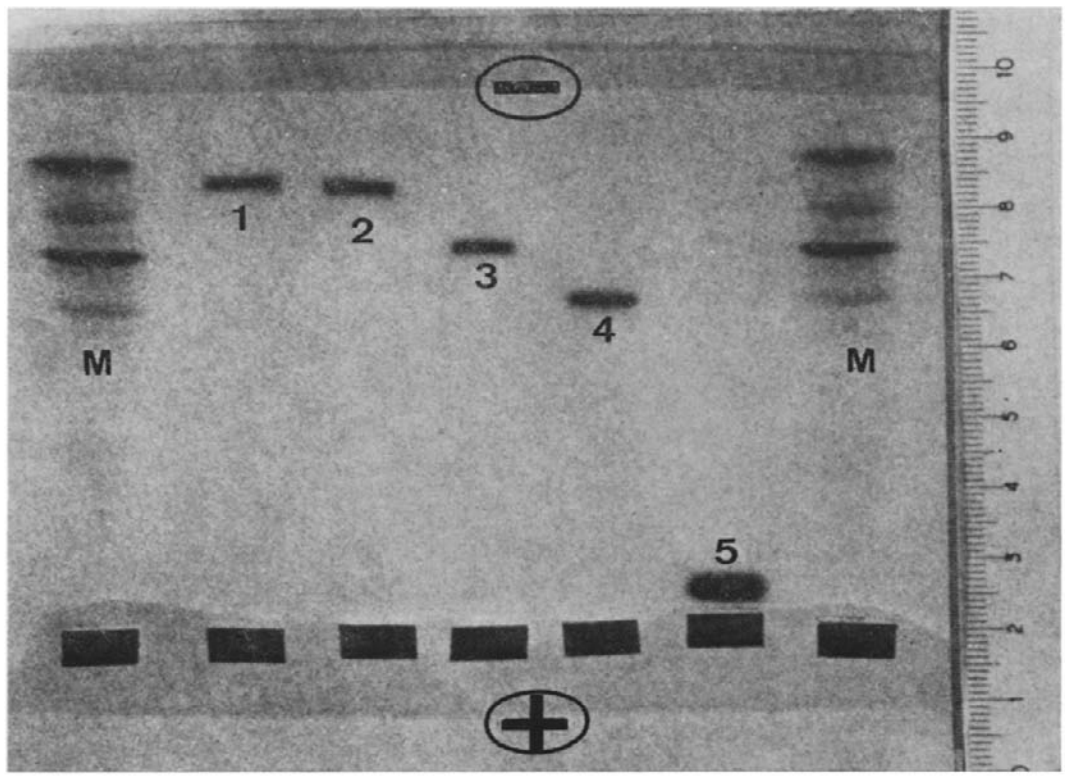

Fig. 2. Isoelectric focusing of peptides listed in Table I with MW < 1000. M = pH markers (whale myoglobin, horse myoglobin, β-lactoglobulin A and B).

Staining procedure

After removal from the apparatus, the gel was stained with the following modified Pauly's reagent[3]:

1 part stock solution I (0.4 *M* sulphanilic acid in water);
1 part stock solution II (0.4 *M* sodium nitrite in water);
1 part stock solution III (2.0 *N* HCl);
7 parts water;
10 parts stock solution IV (2.0 *N* sodium carbonate).

Stock solutions were kept at 4°.

Red peptide bands were observed following immersion of the gel in this staining solution.

Another procedure was used for histidine-containing peptides of higher molecular weight (MW $\geqslant$ 3000) and histidine-containing proteins. The peptides were first fixed by immersion of the gel in 15% trichloroacetic acid for *ca.* 3 h followed by rinsing with 2 *N* Na_2CO_3 and staining in the usual manner.

RESULTS AND DISCUSSION

Figs. 2 and 3 show the results of applying this staining technique to low

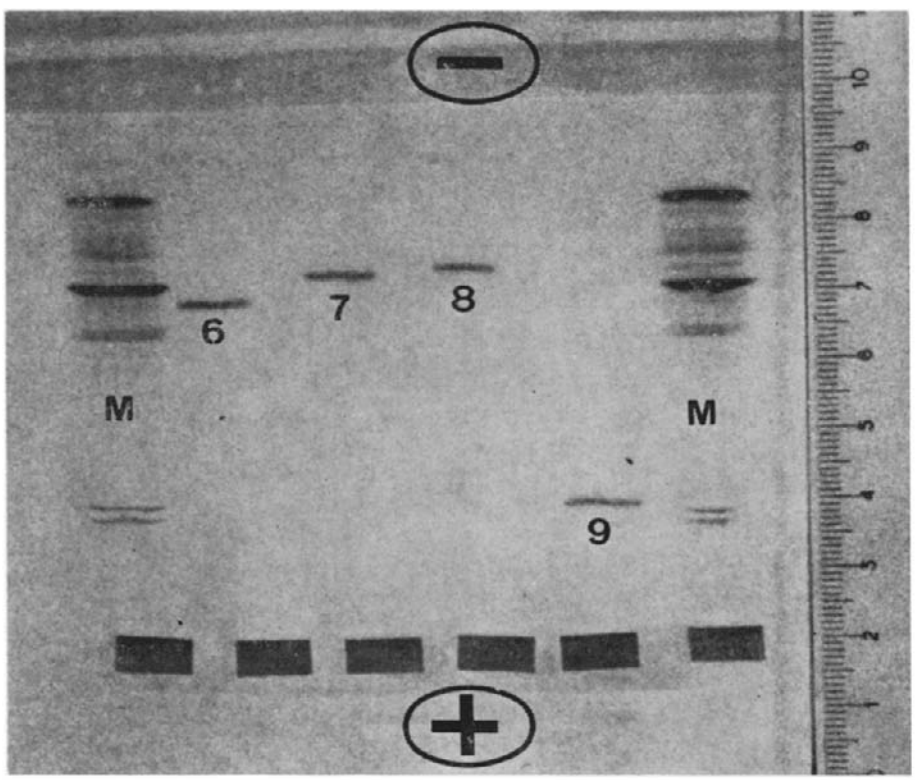

Fig. 3. Isoelectric focusing of peptides listed in Table I with MW > 1000. M = pH markers (whale myoglobin, horse myoglobin, β-lactoglobulin A and B).

molecular weight, histidine-containing peptides, and demonstrate that the method gives a satisfactory staining intensity permitting accurate determination of the isoelectric points (p*I*).

The observation that the staining mixture does not react with ampholines is of particular interest.

In order to determine the p*I* values, small pieces of gel can be placed in a test tube and, after diffusion into 0.5 ml of distilled water, the pH is determined using a microelectrode. As an alternative to pH measurements along the length of the gel, a calibrated mixture of pH markers may be used[4]. Fig. 4 shows the pH range curve and the isoelectric points of the pH markers.

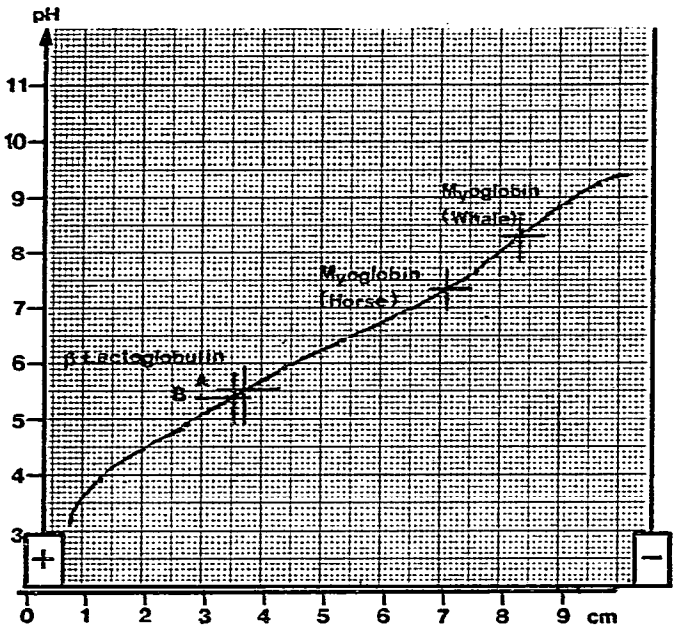

Fig. 4. pH range curve of the higher molecular weight (MW > 1000) peptides and isoelectric points of pH markers.

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