SYSTEMS OF DISC ELECTROPHORESIS FOR STUDYING ACID PROTEINASES

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Alkaline systems of buffers for disc electrophoresis in polyacrylamide gel have been developed in detail. However, in the investigation of some proteins with a low isoelectric point (such as acid proteinases) disc electrophoresis in an alkaline medium frequently does not give satisfactory results because of the "leveling out" of the charges of the proteins (which possess a large number of carboxy groups) and also because of the unfavorable action of an alkaline medium on the activity and structure of labile acid proteinases. In a number of cases, the closeness of the isoelectric points of the enzymes to the pH of separation of the corresponding system (for example, a system with a pH of separation of 3.8) likewise unfortunately does not permit the effective use of such a system for electrophoresis in an acid medium.

Taking the recommendations in the literature into account [1, 2] we have developed and tested systems with pHs of separation of 5.5 and 5.2. For comparison, experiments were performed with systems having pH values of 3.8 [3] and 6.0 [4] and with the widely used alkaline system having pH 9.5 [5]. The experimental part of the paper gives the compositions of the corresponding solutions for performing disc electrophoresis in a system with a pH of separation of 5.5 by means of which it was possible to obtain the best results in the investigation of the acid proteinases.

Disc electrophoresis was used to investigate chromatographically pure porcine pepsin, awamorin (an acid proteinase from Aspergillus awamori) [6], and DNHA-pepsin* [7].

In spite of the fact that in systems with pHs of separation of 3.8, 5.2, 6.0, and 9.5 only one band was detected in native pepsin, the preparation was shown by disc electrophoresis at pH 5.5 to be heterogeneous and under these conditions it separated into two components – a main component and a minor one (Figs. 1 and 2). In the same system it was possible likewise to detect three components in DNHA-pepsin (Fig. 3B) that had not been detected in the investigation of the protein in other systems. The results of the disc electrophoresis of pepsin and of DNHA-pepsin at a pH of separation of 5.5 were confirmed by those of isoelectric focusing in pH gradients 1-3 and 3-5, respectively. As can be seen from Fig. 4. native pepsin consists of two components (with isoelectric points of 2.07 and 2.75) and DNHA-pepsin of three components (with isoelectric points of 3.05, 3.94, and 4.23). It must be mentioned that the amount of the minor component with the isoelectric point of 2.75 was only 15% of the total protein of the sample.

As further investigations of this component showed, it was indistinguishable from the pepsin with an isoelectric point of 2.07 in its specific activity with respect to hemoglobin, had an isoleucine residue at the N-end of the molecule, contained one mole of inorganic phosphorus per mole of protein, and its amino-acid composition was close to or identical with that of porcine pepsin A (a pepsin with an isoelectric point of 2.07). As disc electrophoresis in the presence of sodium dodecyl sulfate showed, the molecular weights of the two components of the pepsin preparation were the same or differed only very slightly (by less than 400-500 daltons) [8]. The system with a pH of separation of 5.5 proved to be extremely convenient for checking the homogeneity of the awamorin during its purification (Fig. 3A, B).

*DNHA-pepsin is pepsin treated at pH 5.5 with mono-N-dinitrophenylhexamethylenediamine in the presence of N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide.

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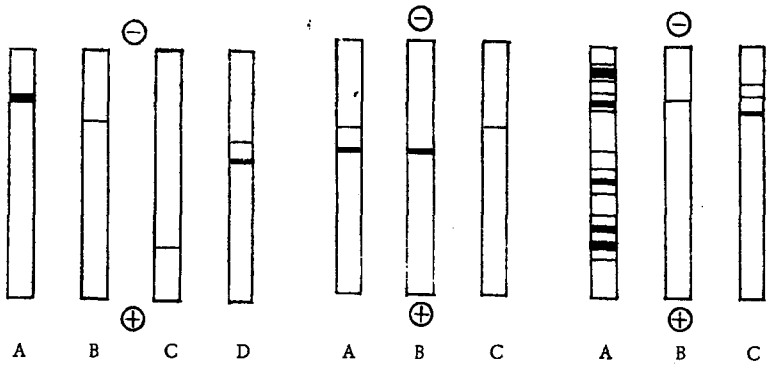

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Results of the disc electrophoresis of porcine pepsin in buffer systems with different pH values of separation: A) 3.8; B) 5.2; C) 9.5; D) 5.5.

Fig. 2. Results of the disc electrophoresis of the two forms of pepsin in a buffer system with a pH of separation of 5.5: A) initial pepsin preparation (before isoelectric focusing); B) pepsin with an isoeletric point of 2.07; C) pepsin with an isoelectric point of 2.75.

Fig. 3. Results of the disc electrophoresis of awamorin and of DNHA-pepsin in a buffer system with a pH of separation of 5.5: A) crude preparation of awamorin; B) highly purified preparation of awamorin; C) DNHA-pepsin.

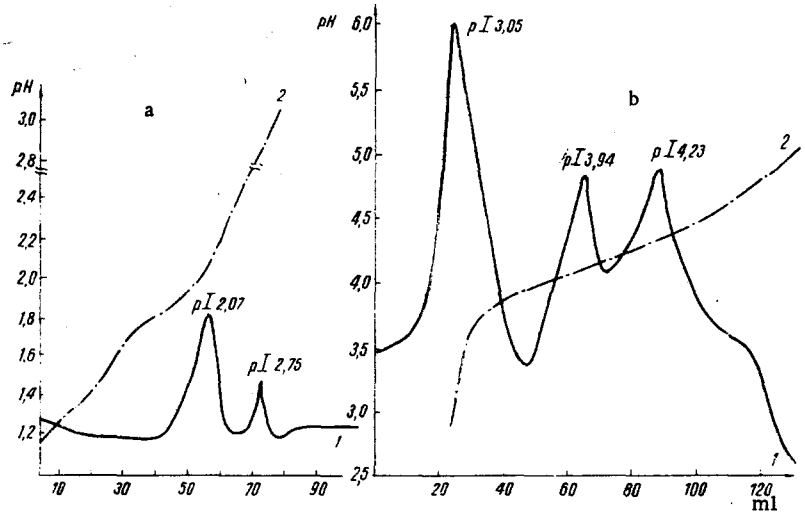

Fig. 4. Results of the isoelectric focusing of porcine pepsin in a pH 1-3 gradient (a) and of DNHA-pepsin in a pH 3-5 gradient (b): 1) E_{280}; 2) pH gradient.

Since the separating capacity of a system with a pH of separation of 5.5 is considerably higher than the separating capacity of a number of systems for disc electrophoresis used at the present time, this system can, in our opinion, be recommended for the electrophoretic investigation of acidic proteins.

EXPERIMENTAL

In the performance of the experiments in a system with a pH of separation of 5.5, we used the following solutions: solution A containing 4.106 M β-alanine ("Reanal," Hungarian Peoples' Republic) and 0.426 M hydrochloric acid; solution B – 0.27 M hydrochloric acid and 0.31 M β-alanine; solution C – 30% of acrylamide ("Koch-Light," Great Britain), and 1.5% of N,N'-methylenebisacrylamide ("Reanal"); solution D –

0.004% of riboflavin ("Reanal"); solution E – 2% of ammonium persulfate ("Reanal"); and 0.015% of TEMED* ("Reanal"); and solution F – 0.0075% of TEMED; the upper electrode buffer was 0.04 M cacodylic acid ("Schuchardt," German Federal Republic) and 0.05 M β-analine, and the lower electrode buffer was 0.05 M hydrochloric acid and 0.06 M β-alanine. Composition of the microporous 12% lower gel: four parts of solution C, 2.5 parts of solution A, and 3.5 parts of solution E. The macroporous 4.5% upper gel consisted of 1.5 part of solution C, 2.5 parts of solution B, two parts of solution D, and four parts of solution F. The polymerization of the gels in tubes with a size of 5 × 60 mm took about 15 min. On each gel was deposited 10-50 μg of protein in admixture with 20% sucrose. Electrophoresis was carried out for 30-45 min at a current strength of 5 mA/gel and a voltage of 300-400 V (in the first 5 min, at 1.5 mA/gel and a voltage not exceeding 100 V). The course of the electrophoresis was followed by the movement of the clearly visible electrophoretic front. The staining and clearing of the gels was performed as described previously [9].

The isoelectric focusing of the DNHA-pepsin was performed in a 2% solution of Ampholines pH 3-5 by the method of Vesterberg and Svensson with the addition to the solutions for focusing of the nonionic detergent "Brij 35" ("Schuchardt," German Federal Republic) [10]. At a temperature of 4°C and a voltage of 500-600 V, focusing took 80-90 h. After the end of the experiment, 2-ml fractions were collected and in them were determined the pH (at 4°C), the absorption at 280 nm, and the activity of the protein with respect to hemoglobin.

The isoelectric focusing of the native procine pepsin was performed in a pH 1-3 gradient by Pettersson's method [11] at 2°C with a voltage of 150-200 V for 120-140 h.

CONCLUSIONS

A system of buffers for the separation of acid proteinases by disc electrophoresis has been developed.

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*TEMED is N,N,N',N'-tetramethylethylenediamine.