Separation of the Similarly Charged Iso-enzymes of *Astacus leptodactylus* Trypsin for Quantitative Amino Acid Analysis and Specificity Studies

The introduction of disc electrophoresis by ORNSTEIN and DAVIS[1] made available a quality of resolution unknown until then. The practicability of this method for analytical purposes is demonstrated by its ample use. But the recovery of isolated protein usually requires rather complicated apparatus. It should therefore be emphasized that it is possible to obtain by disc electrophoresis, without any special equipment, protein fractions large enough to carry out most of the analyses requiring high-purity protein, such as quantitative amino acid analysis or studies on catalytic properties. It was only by the procedure described below that we succeeded in establishing the cleavage specificity of certain proteases, because it was extremely difficult to purify the preparations from all traces of exopeptidase contaminants[2].

In the course of our investigations on the evolution of invertebrate endopeptidases[3,4], we encountered the necessity to analyze the 2 isoenzyme bands of the trypsin-like protease of the crayfish *Astacus leptodactylus* separately. A preparation of this enzyme purified by repeated gel-filtration and anion exchange chromatography, though without other detectable contaminants, still contained both isoenzyme bands (Figure, b). They being separable by no other means, we established the quantitative amino acid analysis for each band[5] by the following procedure: 1.5 mg of the purified protein were applied to the 8 tubes (5 mm in diameter, 75 mm in length) of a Shandon analytical disc electrophoresis apparatus, i.e., each gel contained approximately 200 µg of protein. In regard to the subsequent estimation of amino acid content, the use of buffers containing amino acids must be avoided. We therefore used a modified diethyl barbituric acid *tris*-buffer[6], in which the isoenzyme bands migrated to the anode. Our best results we had with a running buffer of pH 8.2 and gel systems containing 15% acrylamide (W/V).

After the run the gels were rimmed and without fixation of protein bands transferred into an aqueous methylene blue B solution (0.05%, pH 8.0). For staining in acidic medium, methylene blue may be substituted by coomassie brilliant blue or other protein dyes. Within 2 to 5 min protein bands were stained selectively and the clearly visible fractions were cut out with a razor blade. As staining is limited to a very thin ring on the outer surface of the gel, the stained area is cut off and the remaining unstained portion contains the native and active enzyme in a state of very high purity. All 'upper bands' as well as all 'lower bands' were combined, homogenized with a glass rod, and 2 ml of phosphate-buffer (pH 8.0) were added to each fraction. During storage overnight the protein is eluted by diffusion. This step should be repeated. After centrifugation the supernatant is decanted and lyophilized. 2–3 mg of starting material were sufficient to carry out the amino acid analysis of both isoenzyme bands.

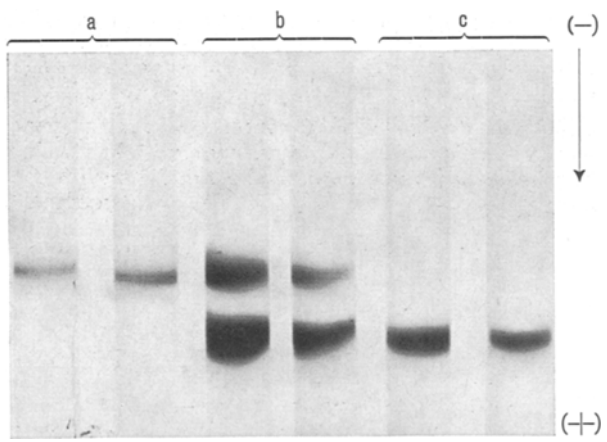
A re-examination of the separated isoenzyme bands by disc electrophoresis under the same conditions as described above proved that they were absolutely pure without contaminants from one fraction to the other (Figure, a–c). Thus the accuracy of amino acid analysis of a protein gained by this method is not limited by the purity of the preparation but rather by the analysis itself.

Another field where high-purity protein is indispensable is the investigation of catalytic properties of an enzyme. We successfully studied in the past the cleavage specificity with peptide substrates of several invertebrate proteases purified by the described procedure[2]. Whereas even the purest preparations obtained by other methods exhibited regularly a sufficient amount of proteolytic impurities, especially of exopeptidases, to prevent clear results, enzyme fractions prepared by the method described never showed additional activities[7].

Zusammenfassung. Durch Anwendung einer selektiven Proteinfärbung in der Disk-Elektrophorese, welche die zu trennenden Banden sichtbar macht, ohne sie zu denaturieren, gelang es, die beiden Isoenzyme der trypsinähnlichen Protease aus dem Krebs *Astacus leptodactylus* vollständig voneinander abzutrennen, obwohl sie sehr ähnliche Ladungen haben. Die erzielte Trennschärfe war eine Voraussetzung für die quantitative Aminosäuren-Analyse sowie für Untersuchungen zur Spaltungsspezifität.

R. ZWILLING

Department of Biochemistry,
Ruhr-Universität Bochum,
D-463 Bochum (Germany), 18 November 1969.



Micro-preparative separation of the 2 similarly charged isoenzymes of the trypsin-like crayfish protease from *Astacus leptodactylus*. (a) Purified 'upper band'; (b) before separation: the fraction contains both isoenzymes; (c) purified 'lower band'.

[1] L. ORNSTEIN and B. J. DAVIS, in *Disc Electrophoresis* (Distillation Products Inc., Rochester, N.Y. 1962). – L. ORNSTEIN, Ann. N.Y. Acad Sci. *121*, 321 (1964). – B. J. DAVIS, Ann. N.Y. Acad. Sci. *121*, 404 (1964).

[2] H.-H. SONNEBORN, R. ZWILLING and G. PFLEIDERER, Z. physiol. Chem., *350*, 1097 (1969).

[3] G. PFLEIDERER, R. ZWILLING and H.-H. SONNEBORN, Z. physiol. Chem. *348*, 1319 (1967).

[4] R. ZWILLING, G. PFLEIDERER, H.-H. SONNEBORN, V. KRAFT and I. STUCKY, Comp. Biochem. Physiol. *28*, 1275 (1969).

[5] R. ZWILLING, in preparation.

[6] R. MAURER, *Disk Elektrophorese* (Walther de Gruyter Co., Berlin 1968).

[7] Acknowledgements. My thanks are due to Mrs. I. FALDERBAUM for skilful technical assistance.