Isoelectric Focusing of Neuraminidase

Although several reports concerning the characterization of neuraminidases of different origin have been published, to our knowledge, the isoelectric point of these enzymes is still unknown. We describe here results of attempts to determine the isoelectric points of *Vibrio cholerae*, *Clostridium perfringens* and influenza virus neuraminidases.

Materials and methods. The bacterial neuraminidases were commercial products obtained from Calbiochem (Los Angeles, California) and Worthington Biochemical Corporation (Freehold, New Jersey), respectively. Influenza virus neuraminidases were prepared from the A_2/Aichi/68 and B/Mass/66 viruses as described by SETO et al.[1], except that neuraminidase and pronase were separated by gel filtration on Sephadex G-100. K 25/45 columns (Pharmacia, Uppsala, Sweden) filled with the Sephadex were used. Fractions of 5 ml were collected using 0.01 *M* *Tris*-HCl buffer-0.14 *M* NaCl (pH 7.0) for elution.

Isoelectric focusing[2] was performed in the LKB 8101 ampholine column (LKB Produkter AB, Bromma, Sweden) for 48–72 h at 200 V. An LKB carrier ampholyte covering the pH range of 3–10 in a glycerol (0–45%, v/v) density gradient was used. Samples of 2 ml each were collected at a speed of 2 ml/min after the electrofocusing process was terminated. The pH of the samples was measured with the Radiometer pH-meter 26 (Radiometer, Copenhagen, Denmark). $^1/_{10}$ ml of 2% bovine γ-globulin (Pentex, Inc., Kankakee, Illinois) in phosphate-buffered saline (pH 7.0), and subsequently 3 ml of a saturated solution of $(NH_4)_2SO_4$ (adjusted to pH 7.0), were added to each fraction. The precipitates were pelleted by centrifugation, dissolved in 0.01 *M* *Tris*-HCl buffer, pH 7.0 (TB; 1 ml), reprecipitated with 1 ml of saturated $(NH_4)_2SO_4$, and after additional centrifugation, finally dissolved in 1 ml of TB. In this procedure glycerol which interferes in the determination of neuraminidase, was removed from the enzyme.

Neuraminidase was determined at pH 7.0 according to the method of AMINOFF[3] using N-acetyl-neuraminyl-lactose as substrate. For the determination of pronase, 'Azocoll' (Calbiochem) was used as a substrate and the procedure recommended by the supplier was followed.

Results and discussion. The 2 influenza virus neuraminidases were completely separated from pronase by gel filtration on Sephadex G-100 (Figure 1) and displayed a considerable heterogeneity upon isoelectric focusing (Figure 2). They were also more basic than the *V. cholerae* and *Cl. perfringens* neuraminidases which appeared to be homogeneous and had isoelectric points of 4.80 and 4.95, respectively. The heterogeneity of the influenza virus enzymes was confirmed in repeated experiments and was not an artefact due to treatment of the viruses with pronase, since enzymes released from the viruses by detergents[4] or organic solvents[5,6] displayed a similar heterogeneity. Neuraminidase isolated from a certain region of the pH gradient appeared again in the same region upon reelectrofocusing. However, A_2/Aichi/68 neuraminidase, recovered from the top of the pH gradient (pH > 10, i.e. beyond the fractionation range of the ampholyte), upon reelectrofocusing became distributed in the pH range of 4.5–10.0 in a pattern shown on Figure 2 (top). This neuraminidase was apparently trapped in the upper electrode (cathode) solution (with no ampholyte present) at the beginning of the electrofocusing process. Disc electrophoresis also revealed the heterogeneity of influenza virus neuraminidase[7]. Unlike the *V. cholerae* and *Cl. perfringens* neuraminidase, the influenza virus

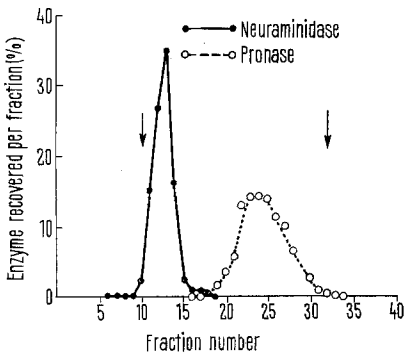

Fig. 1. Distribution of A_2/Aichi/68 influenza virus neuraminidase and pronase in fractions after gel filtration on Sephadex G-100. The left and right arrows indicate the positions of the respective peaks of calf thymus DNA (= void volume) and NaCl when chromatographed separately on the same column.

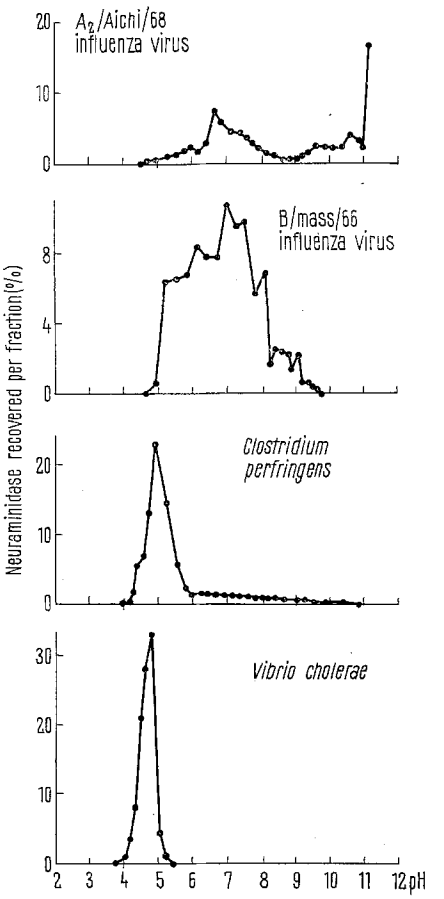

Fig. 2. Isoelectric focusing of neuraminidases from *V. cholerae*, *Cl. perfringens* and from the A_2/Aichi/68 and B/Mass/66 influenza viruses.

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enzymes seem to consist of isoenzymes, as was recently shown for pneumococcal neuraminidase by other methods[8].

Attempts to characterize the neuraminidases of parainfluenza viruses by isoelectric focusing were unsuccessful because a considerable loss in enzyme activity was encountered in the purification process. Similar difficulties have been previously reported[9].

The results reported here explain why *V. cholerae* neuraminidase is less sensitive than the *Cl. perfringens* enzyme to inhibition by polyanions at pH 5, and why influenza virus neuraminidases are much more readily inhibited even at pH values higher than 5[10], under conditions when they must have a net positive electric charge as shown here. The isoelectric point of influenza virus neuraminidase is relatively high, which is consistent with the low electrophoretic mobility (at pH 8.9) of the enzyme compared with that of other influenza virus envelope proteins[4,11].

Zusammenfassung. Die isoelektrischen Punkte der Neuraminidasen von *Vibrio cholerae* (pH 4.80) und *Clostridium perfringens* (pH 4.95) wurden mittels isoelektrischer Fokusierung bestimmt. Neuraminidasen zwei verschiedener Influenzaviren (A_2/Aichi/68 und B/Mass/66) wurden entsprechend analysiert. Die Virus-Neuraminidasen waren Heterogen und hatten wesentlich höhere isoelektrische Punkte als die bakteriellen Enzyme.

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The Prosthetic Group of the Green Chromoprotein of *Patella* Ova

The colour of the mature ova of limpets varies from dark brown to olive-green[1]. A green chromoprotein has been isolated from ripe olive-green ovaries of *Patella caerulea*[2], but the nature of the prosthetic group has not been elucidated[2,3]. The present communication describes the identification of the prosthetic group as a close derivative of chlorophyll *a*.

Patella vulgata were collected at Roscoff in France and *Patella caerulea* were collected in Malta. The main green pigment in ova of *Patella vulgata* was isolated in the form of a methyl ester which proved to be methyl phaeophorbide *a*. The ripe ova were dried in vacuo and extracted with methanolic HCl (6–8%, w/w) for 72 h at 0 °C. The dark green extract was transferred to chloroform and submitted to thin-layer chromatography on silica gel G. A fast moving green pigment (Rf 70–80) was isolated with chloroform-*n*-butanol (190:10, v/v) and purified with carbon tetrachloride-methyl acetate (100:50, v/v) as solvents. The pigment showed absorption maxima at 274, 326, 418, 475, 512, 542, 565, 612 and 668 nm and a fluorescence maximum at 670 nm. It behaved as an authentic sample of methyl phaeophorbide *a* in thin-layer chromatography.

Green chromoprotein was isolated from the ova of *Patella caerulea* as described previously[2]. The chromoprotein showed absorption maxima at 395, 585 and 625 nm in 0.1 *M* ammonium bicarbonate (pH 8). A 0.5% solution of the chromoprotein in 0.1 *M* ammonium bicarbonate was digested with trypsin (0.1 mg/ml) at 15 °C for 48 h. The solution became yellow with a greenish tinge and showed a large absorption maximum at 410 nm, two smaller maxima at 610 and 665 nm, and an inflexion at 540 nm. A pigment with similar absorption was obtained by refluxing lyophilized chromoprotein with boiling methanol for 8 h. This pigment was esterified with 14% boron trifluoride in methanol, transferred to chloroform and purified by thin-layer chromatography on silica gel G with carbon tetrachloride-methyl acetate (100:50, v/v) as solvent. A fast moving yellowish green pigment (Rf 80) was obtained. The pigment showed absorption maxima at 276, 319, 355, 385, 416, 505, 535, 560, 605 and 660 nm in dioxan. The absorption spectrum showed the features of a chlorin[4] and the IR-spectrum showed a peak in the C=C stretching region at about 1625 cm^{-1} which is found in chlorins[5]. The pigment was identified as chlorin a_6 methyl ester in view of the preparation of methyl phaeophorbide *a* from the ova. It is probable that the isocyclic ring was broken during the extraction of the pigment from the chromoprotein with boiling methanol.

The present findings would appear to show that the prosthetic group of the green chromoprotein of *Patella* ova is a close derivative of chlorophyll *a*, namely, phaeophytin a_5 or phaeophorbin a_5. This provides a simple explanation for the reversible yellow colour change of the chromoprotein at alkaline pH (see [2]) in terms of enolization of the hydrogen atom at carbon C-10 to the carbonyl group at carbon C-9 as in the ether-methanolic KOH phase test for chlorophylls[6].

Résumé. Les ovaires des Patelles *Patella vulgata* et *Patella caerulea* contiennent un chromoprotéide vert qui a été précédemment étudié. Dans le présent travail, le groupe prosthétique de ce pigment est identifié. Il s'agit d'un tètrapyrrole dont la structure est proche de celle de la chlorophylle *a*. L'identification repose sur l'isolement des esters méthyliques du phéophorbide *a* d'une part (à partir des gonades entières) et de la chlorine a_6 d'autre part (à partir du chromoprotéide).

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