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K_2-TARTARATE GRADIENT CENTRIFUGATION IN THE ISOLATION OF PURIFIED INFLUENZA VIRUS HAEMAGGLUTININ AFTER BROMELAIN CLEAVAGE

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Summary. — A method of haemagglutinin (HA) purification by means of K_2-tartarate gradient centrifugation is described. Different influenza virus strains possessing the antigenic formulas H1N1, H2N2 and H3N2 yielded pure and immunologically active HA samples.

Key words: haemagglutinin; influenza virus; bromelain cleavage; K_2-tartarate gradient; electron microscopy

Introduction

The surface antigens HA and neuraminidase (NA) of the majority of influenza virus strains can be loosened from virus particles by proteolytic enzymes. The attempts of Brand and Skehel (1972) to obtain pure HA were based on the assumption that bromelain used for envelope cleavage would destroy the NA so that purified HA could be obtained after sucrose gradient centrifugation. In contrast, Hoyle and Almeida (1971) stated that HA and NA sticked firmly together. Due to their similar molecular mass, they cannot be distinctly separated in sucrose gradients. No NA digestion had been observed in our trials aimed at obtaining the pure HA by bromelain cleavage, despite of the use of enzyme probes of different origin. For separation of the cleaved virion components, instead of the sucrose gradient, the K_2-tartarate gradient was employed as recommended by Compans *et al.* (1970). By this way, a clear-cut separation of the HA and NA was achieved. This paper describes in detail the methodic application of the K_2-tartarate gradient for isolation of HA. In our experiments with 12 different influenza virus strains pure HA was always obtained.

Materials and Methods

Viruses. The following influenza virus strains were grown in embryonated eggs: A/PR/8/34 (H1N1), A/FM1/47 (H1N1), A/USSR/90/77 (H1N1), NIB6 (A/USSR/90/77 + A/PR8/34) (H1N1), A/Brazil 11/78 (H1N1), X-71 (A/Brazil 11/78 + A/PR8/34) (H1N1), A/Singapore/1/57 (H2N2) A/Hongkong 1/68 (H3N2), MRC11 (A/Pt. Chalmers 1/73 + A/PR8/34) (H3N2), X-47 (A/Victoria 3/75 + A/PR8/34) (H3N2), NIB4 (A/England 321/77 + A/PR8/34) (H3N2), X-73 (A/Bangkok 1/79 + A/PR8/34) (H3N2) and B/Hongkong 5/72). The virus-containing allantoic fluids were precipitated with 10% v/v of saturated ammonium sulphate (AS). After 30 min

incubation, the solution was thoroughly shaken, the precipitate was removed by centrifugation and the supernatant was concentrated by repeated differential centrifugation.

Bromelain treatment was performed according to Brand and Skehel (1972). The following bromelain preparations were used: bromelain from pineapple stems (Ferak, Berlin West); bromelain from pineapple stems (Serva GmbH Co, Heidelberg, ca 1200 GDU); bromelain grade II (Sigma, Chemical Co. St. Louis, Miss., U.S.A.)

Density gradient centrifugation was performed in ultracentrifuge VAC 601 (VEB Zentrifugenbau Engelsdorf, G.D.R.) equipped with rotors W6 × 85, SW 3 × 5 and SW 3 × 35. Linear gradients: saccharose 5—40% (ρ = 1.02—1.08) and K_2-tartarate 5—40% (ρ = 1.025—1.305). Centrifugation times: 4, 7 and 18 hr. Acceleration: 100, 000 × g. Handling of the gradients: 5 ml gradient was divided into 12 fractions, 35 ml gradient into 24 fractions; HA content, NA activity and protein content were determined in each fraction.

HA was tested by the following methods:

— HA units (HAU were determined according to Mayrd *et al.* (1974)

— quantitation of HA activity (μg/ml) in single radial diffusion (SRD) according to Schild *et al.* (1975)

— HA detection by immunodiffusion test (Ouchterlony, 1965).

NA activity was estimated according to Aymard-Henry *et al.* (1973) using ovomucoid (Leithold, Kleinmachow, G.D.R.) substrate instead of fetuin.

Protein content was determined according to Lowry *et al.* (1951) using BSA as standard (measured at 280 nm, Spektralphotometer VSU2, C. Zeiss, Jena).

Polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). The gels were stained with Coomassie brilliant blue G-250 (0.5% solution) and OD was evaluated in a gel-scanner (1310, ISCO, U.S.A.)

Specimens contrasted with phosphotungstic acid were viewed in Tesla BS 500 electron microscope.

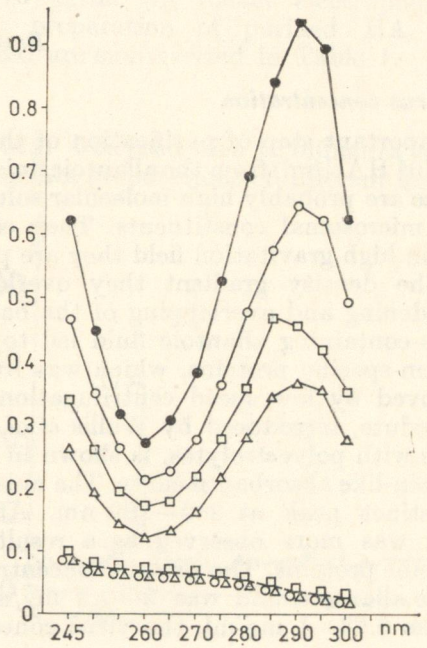

Fig. 1.

UV-absorbance of virus-containing crude allantoic fluid before and after AS treatment

Strains: ●——● A/PR8/34; △——△ A/FM/47 (H1N1); ○——○ A/Singapore 1/57 (H2N2); □——□ A/Hongkong 1/68 (H3N2).

Before AS precipitation: full line; after AS precipitation: interrupted line.

Abscissa: nm; ordinate: UV-absorbance (%).

Table 1. Characterization of virus strains and their concentrates

Virus strain	Antigenic formula	Content (mg/ml)	HA content (HA/ml)	NA activity (released NANA, mg × 10^2/ml)
A/PR8/34	H1N1	36	10^6	1.2
A/FM1/47	H1N1	41	10^6	41.3
A/USSR 90/77	H1N1	26	10^6	7.2
NIB 6	H1N1	29	10^6	1.6
A/Brazil 11/78	H1N1	35	10^6	3.0
X-71	H1N1	27	10^6	10.0
A/Singapore 1/57	H2N2	29	10^6	100.0
A/Hongkong 1/68	H3N2	40	10^6	290.0
MRC11	H3N2	27.5	10^6	100.0
X-47	H3N2	24	10^6	21.5
NIB 4	H3N2	25	10^6	14.6
X-73	H3N2	29	10^6	11.0
B/Hongkong 5/72		32	10^6	35.0

Results

Virus concentration

Important step of purification of the highly concentrated influenza virus ($\geqq 10^6$ HAU/ml) from the allantoic fluid is the removal of non-specific proteins. These are probably high molecular soluble proteins in addition to cell debris and microsomal constituents. They can be precipitated in the cold (4 °C) and in high gravitation field they are pelleted along with the virus particles. In the density gradient they overload the separation capacity causing broadening and overlapping of the bands. The addition of saturated AS to virus-containing allantoic fluid led to formation of a fine flaky precipitate of non-specific proteins, which was free of any viral activity and could be removed by low speed centrifugation. An example of such pre-purification procedure, introduced by Wallis *et al.* (1972) for concentration of influenza virus with polyectrolytes, is shown in Fig. 1. The results revealed a typical protein-like absorbance curve. The untreated crude allantoic fluid represented a distinct peak at 280—290 nm. After partial precipitation with AS no peak was more observed as a result of a profound elimination of non-specific proteins. The protein concentration according to Lowry in untreated crude allantoic fluid was 2—2.2 mg/ml, whereas in the AS-treated fluids it was 0.92—1 mg/ml, the virus concentration remaining unchanged.

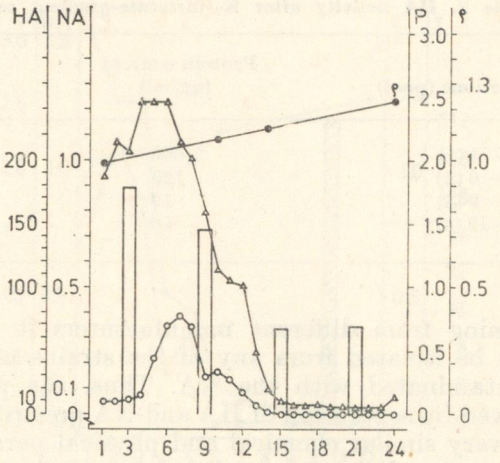

Fig. 2.
Isolation of monospecific HA in K_2-tartarate gradient
Strain: NIB 4 (H3N2)
NA activity ○——○; protein content △——△; density ●——●; columns: HA activity (6 fractions were pooled, the HA activity determined in μg/ml; protein content of fractions 19—24 was measured according to Lowry). Abscissa: fraction number; ordinates: HA (μg/ml), NA (absorbance at 594 nm), P (protein, absorbance at 280 nm), ρ (density in $g.cm^{-3}$).

The repeated control of HA content (HAU/ml) in the fresh allantoic harvest up to different virus concentration steps clearly showed the advantage of previous AS-treatment. The decrease in titre always occurring at storage of allantoic fluid before further working procedure can be avoided so that, in addition to better purification, the virus yield is considerably enhanced. Viral concentrates containing from 10^6 up to 4×10^6 HAU/ml and ovalbumin below detection level of 70 μg/ml (in rocket electrophoresis) were used as initiation material for preparation of purified HA. The analytic data of the concentrates used are summarized in Table 1.

Preparation of HA

The method of Brand and Skehel (1972) was used with 12 influenza A and 1 influenza B strain. Employing 3 bromelain preparations of different quality

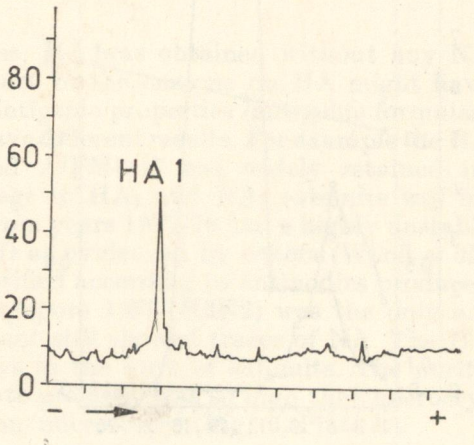

Fig. 3.
Detection of pure HA in PAGE visualized by gel-scanner 1310
Strain: X-73 (H3N2); λ = 660 nm; absorbance 0.5.
Arrow: direction of sample movement. OD = optical density.

Table 2. HA activity after K_2-tartarate-gradient centrifugation of the strain X-73 (H3N2)

Fractions (pool)	Protein content (μg/ml)	HA activity (μg/ml)	NA activity (NANA released μg/ml)
1— 3 (1)	1,000	200	50
4— 6 (2)	120	100	0
7— 9 (3)	12	11	0
10—12 (4)	10	11	0

coming from different manufacturers it was found that pure HA could not be isolated from any of the strains used and that in each case it was contaminated with the NA. Thus, the problem of HA isolation can be solved by separation of HA and NA present in the cleavage product. Because of very similar chemical and physical parameters of envelope proteins, the sucrose gradient seems to be unsuitable for this purpose. The cleavage mixture was concentrated 10 times by vacuum dialysis and layered over a K_2-tartarate gradient run for 7 hr at 100,000 × g. As shown in Fig. 2, this procedure succeeded in isolation of HA not contaminated with NA. The gradient fractions were tested for HA and NA activities and for protein content: the HA activity (μg/ml) by means of SRD was determined always in 6 pooled fractions. While the fractions 1—6, 7—12 and 13—18 still appeared to be HA and NA mixtures, the fractions 19—24 contained purified HA as evidenced by the coincidence of SRD-mediated HA activity with the protein content according to Lowry. A particularly high and pure HA yield was obtained when the Bangkok X-73 (H3N2) recombinant had been used. With this strain, no NA could be found already in pool 2 of the K_2-tartarate gradient. The

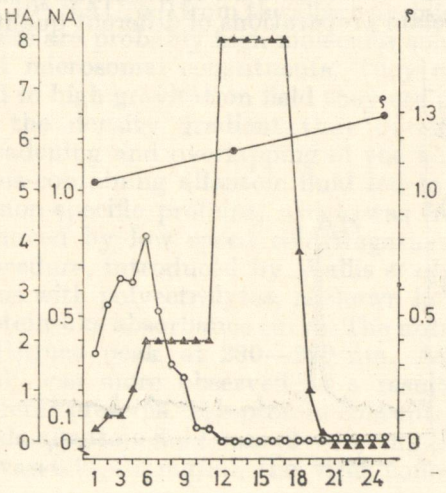

Fig. 4.
Isolation of monospecific HA in K_2-tartarate gradient
Strain: A/PR8/34 (H1N1)
HA content ▲——▲; NA activity ○——○; density ●——●.
Abscissa: fraction number; ordinates: HA (HAU/ml), NA (absorbance at 549 nm), ρ (density, in $g.cm^{-3}$).

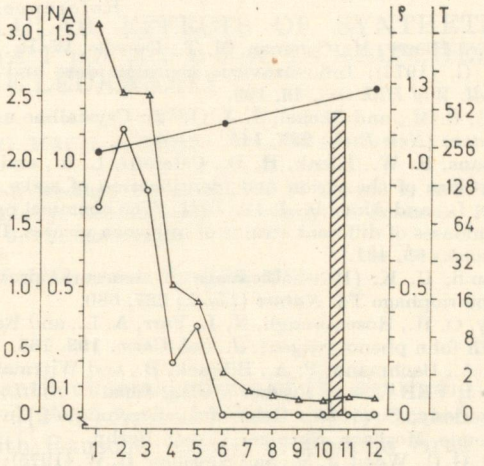

Fig. 5.
Isolation of monospecific HA in the K_2-tartarate gradient
Strain: NIB 6 (H1N1)
NA activity ○——○; protein content △——△; density ●——●. Dashed column: antibody titre of monospecific antiserum in rats (immunization dose: 16 μg protein per animal).
Abscissa: fraction number; ordinates: NA (absorbance at 549 nm), P (protein, absorbance at 280 nm), ρ (density in $g.cm^{-3}$), T (haemagglutination-inhibiting antibody titre).

isolated HA revealed a single peak in PAGE; the minute amount of HA_2 could not be detected with the gel scanner 1310 (ISCO, U.S.A.) used in these experiments (Table 2; Fig. 3).

Discussion

The results of Brand and Skehel (1972) were not confirmed in our work. The proteolytic cleavage of influenza virus with bromelain is considerably influenced by several factors:

— quality and amount of bromelain
— disintegration conditions (time, temperature, mixing)
— virus strain (antigenic formula).

Regardless of the chosen conditions, HA was obtained without any NA contamination. Therefore, the influence of the enzyme on HA might have differed. Virus strains with different antigenic properties (antigenic formulas) under the chosen reaction conditions gave different results. For example the HA isolated from strains A/PR/8/34 and A/FM1/47 has widely retained its activity, what means that the cleavage to HA_1 and HA_2 subunits was incomplete (Fig. 4). The H1N1 strains from years 1977/78 had a highly unstable HA, varying in a broad range in SRD as evidenced by others (Wood *et al.*, 1979). The antigen was therefore identified according to antibodies produced in animals (Fig. 5). The strain A/Singapore 1/57 (H2N2) was the only exception, as the HA in tartarate gradient still showed traces of NA. The HA isolated from H3N2 strains was always in the form of subunits. The purity of the samples obtained in K_2-tartarate gradient was so high that microcrystalls were formed as seen in electron microscopy (Fig. 6, Plate L).

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