1 Y. KAKIHARA AND K. ICHIHARA, *Med. J. Osaka Univ.*, 3 (1953) 497.
2 M. UCHIDA, Y. TAKETOMO, Y. KAKIHARA AND K. ICHIHARA, *Med. J. Osaka Univ.*, 3 (1953) 509.
3 H. YOSHIMATSU, *Med. J. Osaka Univ. Japan. Ed.*, 9 (1957) 727.
4 H. YAMADA, H. KUMAGAI, H. MATSUI, H. OHGISHI AND K. OGATA, *Biochem. Biophys. Res. Commun.*, 33 (1968) 10.
5 H. KUMAGAI, H. YAMADA, H. MATSUI, H. OHKISHI AND K. OGATA, *J. Biol. Chem.*, in the press.
6 H. KUMAGAI, H. YAMADA, H. MATSUI, H. OHKISHI AND K. OGATA, *J. Biol. Chem.*, in the press.
7 H. KUMAGAI, H. MATSUI, H. OHGISHI, K. OGATA, H. YAMADA, T. UENO AND H. FUKAMI, *Biochem. Biophys. Res. Commun.*, 34 (1969) 266.
8 T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
9 A. E. BRAUNSTEIN AND M. M. SHEMYAKIN, *Biokhimiya*, 18 (1953) 393.
10 D. E. METZLER, M. IKAWA AND E. E. SNELL, *J. Am. Chem. Soc.*, 76 (1954) 648.

Received February 13th, 1970

Biochim. Biophys. Acta, 206 (1970) 476–479

BBA 63456

Purification and properties of *N*-acetylneuraminate lyase from beef kidney cortex

The enzyme *N*-acetylneuraminate lyase (*N*-acetylneuraminate pyruvate-lysase, EC 4.1.3.3, also known as neuraminic acid aldolase) isolated from *Clostridium perfringens* was shown by GANTT *et al.*[1] to be selectively inhibited by the substrate analogue 3-fluorosialic acid. Incubation of the enzyme in the presence of this analogue resulted in an analogue concentration-dependent irreversible inactivation. This inactivation was prevented by increasing amounts of the substrate *N*-acetylneuraminic acid. This communication describes the preparation of *N*-acetylneuraminate lyase from a bovine source and its properties, particularly with respect to the effect of 3-fluorosialic acid.

The enzyme was assayed by following the liberation of either pyruvate or *N*-acetylmannosamine resulting from the cleavage of the substrate *N*-acetylneuraminic acid. Each assay mixture contained 0.10 ml of 1.0 M potassium phosphate (pH 7.2), 0.10 ml of 0.10 M *N*-acetylneuraminic acid, 0.050–0.50 ml of enzyme preparation, and distilled water to a total volume of 1.0 ml. After 15-min incubation at 37° the reaction was terminated by heating at 100° for 2 min. Any precipitated protein was removed by centrifugation. The *N*-acetylmannosamine liberated was determined by the modified Morgan–Elson reaction as described by BRUNETTE *et al.*[2]. This assay was used throughout the purification to follow the increase in specific activity of the preparation. Pyruvate formed was determined by the coupled reaction with NADH and lactate dehydrogenase as described by BRUNETTE *et al.*[3]. A unit of activity is defined as the μmoles of either *N*-acetylmannosamine or pyruvate liberated per 15 min at 37°. Protein was determined spectrophotometrically[4]. Specific activity of the enzyme preparation is defined as units/mg protein.

Beef kidneys were obtained fresh from a local slaughterhouse. Unless otherwise stated, all preparative procedures were carried out at 4°, and centrifugations at 14 000 × *g*. A total of 900 g of kidney cortex was homogenized in 1800 ml of distilled

water for 90 sec, and the homogenate was clarified by centrifugation for 60 min. The supernatant (initial extract) solution (1593 ml) was treated with 0.4 vol. of 2% protamine sulfate (pH 7.0), and the inactive precipitate was removed by centrifugation for 25 min. The supernatant from the protamine treatment (2092 ml) was slowly brought to 30% saturation with respect to solid $(NH_4)_2SO_4$. The precipitate was removed by centrifugation for 30 min and discarded. Additional solid $(NH_4)_2SO_4$ was then added to the supernatant to 55% saturation. The precipitate, containing most of the enzyme activity, was collected by centrifugation, dissolved in 209 ml of 0.05 M potassium phosphate (pH 6.8) and dialyzed for 4 h against 4 l of the same buffer. The enzyme solution was then diluted to 418 ml with the same potassium phosphate buffer, and 5-ml portions (in 16 mm × 150 mm test tubes) were heated at 70° for 15 min. The fractions were combined with a 1.0-ml water rinse of each tube and the large inactive precipitate removed by centrifugation. The pale yellow heat supernatant was transferred to a beaker in a −10° bath. After the enzyme solution reached 0°, it was slowly brought to 20% (v/v) with respect to ethanol (precooled to −10°). The precipitate was removed by centrifugation at −10° and discarded. The supernatant solution was brought to 31% (v/v) with respect to ethanol (at −10°) and the precipitate collected as above. This pellet was dissolved in 50 ml of 0.02 M potassium phosphate (pH 6.8) and the enzyme solution applied to a hydroxylapatite column (2.6 cm × 15 cm) which had previously been equilibrated with the same buffer. The active protein was eluted with a linear gradient of 0.02–0.20 M potassium phosphate of pH 6.8 (total volume 400 ml). Fractions (4.0 ml) were collected and assayed for enzymatic activity[2]. The most active fractions (44 through 57) were pooled and concentrated to 13 ml by two successive treatments with dry Sephadex G-25 (coarse grade). This concentrated enzyme solution was then applied to a Sephadex G-200 column (2.2 cm × 72 cm) which had previously been equilibrated with 0.05 M potassium phosphate (pH 7.2) containing 0.10 M KCl. The enzyme was eluted from the column with the same buffer and fractions collected and assayed[2] as before. The most active fractions (33–41) were pooled and frozen at −16°. The purified enzyme usually lost 90% activity within 2 months.

Results of a typical purification are shown in Table I. The fractionation pro-

TABLE I

PURIFICATION OF BOVINE KIDNEY *N*-ACETYLNEURAMINATE LYASE

The units of enzyme activity refer to μmoles of either *N*-acetylmannosamine or pyruvate liberated per 15 min from cleavage of *N*-acetylneuraminic acid.

Fraction	*Total protein (mg)*	*Total enzyme units*	*Specific activity (μmoles/mg protein per 15 min)*	*Purification*	*Recovery of enzyme (%)*
Initial extract	71 000	907	0.013	1	100
Protamine supernatant	33 000	757	0.022	1.7	83.5
30–55% $(NH_4)_2SO_4$ precipitation	12 600	691	0.055	4.2	76.5
Heat treatment supernatant	2 480	578	0.233	17.7	63.6
20–31% ethanol precipitation	740	240	0.324	24.9	37.6
Hydroxylapatite chromatography	25.4	121	4.76	366	13.3
Sephadex G-200 gel filtration	7.06	70.8	10.3	790	7.8

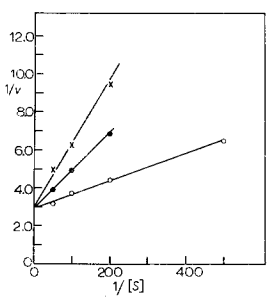
cedures resulted in an almost 800-fold purification with a 7–8% recovery of the total activity. The purified enzyme from beef kidney demonstrated a broad pH activity curve between pH 7.2 and 8.5 and a K_m of 2.4 mM for the substrate *N*-acetylneuraminic acid. In the presence of 0.01 M substrate the enzyme was heat stable for 10 min at temperatures of up to 76°. These properties compare closely to those of the enzyme isolated from other sources[1–3,5].

It has been reported[1] that 3-fluorosialic acid was not a substrate for *N*-acetylneuraminate lyase isolated from *C. perfringens*, as shown by the lack of formation of either of the expected cleavage products fluoropyruvate or *N*-acetylmannosamine. Under similar assay conditions[1], incubation of the 1.0 unit of enzyme from beef kidney for 50 min at 37° in the presence of 10 mM 3-fluorosialic acid showed no detectable liberation of either of these products. Further, in contrast to the results obtained with the enzyme from the bacterial source[1], incubation of 0.11 unit of beef kidney enzyme with 10 mM 3-fluorosialic acid for 60 min at 37° in 0.10 M potassium phosphate (pH 7.2) did not result in any appreciable loss of enzyme activity. Under these conditions the bacterial enzyme[1] was almost completely inactivated by this analogue.

Fig. 1 shows typical Lineweaver–Burk plots demonstrating the effect of the analogue on the rate of cleavage of *N*-acetylneuraminic acid by the beef kidney enzyme. The 3-fluorosialic acid was a competitive type inhibitor of K_i value 9.0 mM.

At final concentrations of 1 mM, Mg^{2+}, Mn^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, and Ca^{2+} had no effect on enzyme activity, while Fe^{3+} and Cu^{2+} inhibited 50%. No enzyme activity could be detected in the presence of 1 mM Hg^{2+}. EDTA at 10 mM had no effect on the activity of the beef kidney enzyme, as was also shown for the bacterial enzyme[1].

The molecular weight of bovine kidney *N*-acetylneuraminate lyase was de-



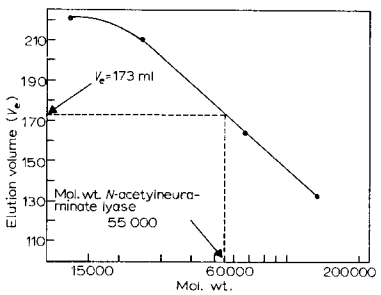


Fig. 1. Effect of 3-fluorosialic acid on reaction velocity (Lineweaver–Burk plot). Initial rates were measured by determining the amount of pyruvate liberated[3] during incubation of 0.11 unit of enzyme under conditions described in the text with the designated concentrations (M) of the substrate *N*-acetylneuraminic acid (○) and either 1 mM (●) or 5 mM (×) 3-fluorosialic acid. Velocity is expressed as μmoles pyruvate liberated per 15 min.

Fig. 2. Molecular weight by Sephadex. A Sephadex G-200 column (2.2 cm × 72 cm) was prepared as described by ANDREWS[6,7] and equilibrated at 4° with 0.05 M potassium phosphate (pH 7.2) containing 0.10 M KCl. A 2.0-ml solution of the following protein standards was used to calibrate the column: 3.0 mg bovine serum albumin (containing dimer), 1.7 mg chymotrypsinogen, and 1.3 mg cytochrome *c*. The molecular weights of these proteins have been tabulated by ANDREWS[7]. Fractions (3.2 ml) were collected and the elution volumes of the proteins plotted *versus* the logarithm of their respective molecular weights. Aggregation of chymotrypsinogen was corrected for as described by ANDREWS[6]. The enzyme molecular weight was determined by adding 4.5 units of enzyme to the standard protein solution and each fraction assayed for *N*-acetylneuraminate lyase activity as described[2]. Protein was estimated by measuring absorbance at 230 nm and cytochrome *c* at 412 nm (ref. 7).

termined by Sephadex G-200 as described in the legend of Fig. 2. The elution volume of the enzyme was determined and the molecular weight value of 55 000 obtained directly from the calibration line in Fig. 2.

This work was supported by Grant CA-3231 from the U.S. Public Health Service, a U.S. Public Health Predoctoral Fellowship (5-F1-Gm-25, 101-03) and U.S. Public Health Service Training Grant 471.

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1 R. GANTT, S. MILLNER AND S. B. BINKLEY, *Biochemistry*, 3 (1964) 1952.
2 P. G. BRUNETTE, W. J. JOURDIAN AND S. ROSEMAN, *J. Biol. Chem.*, 237 (1962) 2447.
3 P. G. BRUNETTE, A. SWANSON AND S. ROSEMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1963, p. 465.
4 H. M. KALCHAR, *J. Biol. Chem.*, 213 (1947) 461.
5 D. G. COMB AND S. ROSEMAN, *J. Biol. Chem.*, 235 (1960) 2537.
6 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
7 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.

Received February 16th, 1970

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