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PRODUCTION OF MICROBIAL NEURAMINIDASES INDUCED BY COLOMINIC ACID

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SUMMARY

Microorganisms capable of producing neuraminidases were sought by using culture media containing colominic acid, a homopolymer of *N*-acetylneuraminic acid, as a sole source of carbon. Among 1000 microorganisms screened, neuraminidases with strong activity and wide specificity were obtained from the culture filtrate of *Sporotrichum schenckii*, *Penicillium urticae*, *Streptomyces* sp., and an unidentified bacterium B 3831-1.

Neuraminidases (sialidases, mucopolysaccharide *N*-acetylneuraminyl hydrolases, EC 3.2.1.18) cleave terminal acyl-substituted neuraminic acid residues from glycoproteins, glycolipids, homopolymers of *N*-acetylneuraminic acid, and oligosaccharides such as *N*-acetylneuramin-lactose. These enzymes have been found in viruses, bacteria, Actinomycetes, protozoa, and vertebrate tissues and plasma.

Among the bacteria secreting neuraminidases are *Vibrio cholerae (comma)* [1–5], *Clostridium perfringens (welchii)* [6–8], *Diplococcus pneumoniae* [9–13], *Corynebacterium diphtheria* [14–16], *Corynebacterium acnes* [17], *Pseudomonas* sp. [18], *Lactobacillus bifidus* [18, 19], *Klebsiella aerogenes* [20], *Erysipelothrix insidosa* [21], *Pasturella multocida* [22–25], some strains of streptococci [10, 26–30], and among Actinomycetes are *Streptomyces albus* [31], *Mycobacteria* sp. [16], and *Nocardia* sp. [16]. Microbial neuraminidases are inducible enzymes and are produced when these microorganisms are grown in the medium such as Todd–Hewitt broth, brain heart infusion, or a medium containing free or bound sialic acid [13, 23, 32, 33].

The present investigation was undertaken to look for the microorganisms which are able to utilize colominic acid, a homopolymer of *N*-acetylneuraminic acid, as a sole source of carbon. Colominic acid has been obtained in improved yields in this laboratory [34]. Neuraminidases with strong activity and wide specificity have come to be obtained from the culture filtrate of fungi, bacteria, and Actinomycetes by using the culture medium containing colominic acid as a source of carbon. This is the first report that has detected the occurrence of neuraminidase in fungi.

The microorganisms used in the present experiments were the strains preserved in this laboratory. Microorganisms isolated from soil, dust, and water were also examined.

The following culture media containing colominic acid as a sole source of

carbon were used throughout this work. The medium for bacteria consisted of 0.5% colominic acid, 0.2% $(NH_4)_2HPO_4$, 0.3% NaCl, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract (pH 7.0). The medium for lactic acid bacteria consisted of 1.0% colominic acid, 0.5% peptone, 0.025% K_2HPO_4, 0.025% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.2% yeast extract (pH 6.8). The medium for yeast contained 1.0% colominic acid, 0.5% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% yeast extract (pH 6.5). The medium for Actinomycetes and fungi consisted of 0.5% colominic acid, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract (pH 6.0).

About 1000 microorganisms including bacteria (280 strains), yeasts (160), fungi (220), and Actinomycetes (340) were grown on the agar slant media as the first screening. Not many microorganisms satisfactorily utilized colominic acid as a sole source of carbon. Among those which gave positive growth were bacteria belonging to *Bacillus*, *Escherichia*, *Aerobacter*, *Pseudomonas*, *Brevibacterium*, and *Staphylococcus*, yeasts belonging to *Hansenula*, *Torulopsis*, *Candida*, and *Rhodotorula*, fungi belonging to *Rhizopus*, *Aspergillus*, *Penicillium*, *Fusarium*, *Ustilago*, *Sporotrichum*, *Corticium*, and *Sclerotinia*, and Actinomycetes belonging to *Streptomyces*, *Mycobacterium*, and *Nocardia*. Active strains which are able to utilize colominic acid were selected and inoculated in 3 ml of liquid medium in a test tube (15 mm diameter). After cultivation for 48 h at 30 °C with shaking (Actinomycetes and fungi were cultivated for 76 h), the neuraminidase activity in the supernatant was determined. Microorganisms which revealed the activity more than about 4 are listed in Table I. *Streptomyces* sp., *Sporo-*

TABLE I

DISTRIBUTION OF NEURAMINIDASE ACTIVITIES AMONG MICROORGANISMS

Incubation mixture for the determination of neuraminidase activity contained 0.1 ml of colominic acid solution (containing 100 μg of colominic acid in 0.1 ml of 0.2 M acetate buffer, pH 4.9), and 0.1 ml of enzyme preparation (culture supernatant). Incubation was conducted for 30 min at 37 °C. Reaction was terminated by adding $NaIO_4$ reagent. N-Acetylneuraminic acid was determined by the thiobarbituric acid procedure of Warren [35]. Enzyme activity was expressed as the amounts (nmoles) of N-acetylneuraminic acid which were released under the reaction conditions described above.

Microorganisms	Activity
Str. griseus IFO 3430	38.2
Str. olivaceus IFO 3409	40.8
Str. scabies IFO 3111	9.7
Streptomyces sp. M 39-1	79.6
Streptomyces sp. MB 53-2	88.7
Streptomyces sp. M 65-3	29.8
Streptomyces sp. MA 89-1	84.8
Streptomyces sp. M 148-1	81.6
Streptomyces sp. M 278-5	60.2
Sp. schenckii IFO 5984	104.9
P. urticae IFO 7011	44.7
A. aerogenes IFO 12010	7.1
B. subtilis IFO 3037	5.8
B. natto AKU 0205	7.8
St. aureus IAM 1099	3.9
Ps. riboflavina IFO 3140	4.5
B 3831-1 (unidentified bacterium)	90.6

TABLE II

COMPARISON OF NEURAMINIDASE ACTIVITIES PRODUCED WITH COLOMINIC ACID MEDIUM AND TODD-HEWITT BROTH

Each microorganism was grown in the medium containing colominic acid as a sole source of carbon (the composition is described in the text) and in Todd-Hewitt broth (Difco) for 76 h (*Actinomycetes* and fungi), or 48 h (bacteria). The reaction mixture contained 0.1 ml of enzyme preparation (culture supernatant), 0.05 ml of 0.2 M buffer solution, and 0.05 ml of substrate solution (colominic acid, 4.0 mg/ml). Incubation was conducted for 10 min at 37 °C and release of *N*-acetylneuraminic acid was compared with that in controls containing the same components but lacking enzyme. The buffer solution used: *Str.* sp., lactate buffer (pH 4.3); *Sp. schenckii*, lactate buffer (pH 3.7); *P. urticae* and Bacterium 3831-1, acetate buffer (pH 4.3). A unit of enzyme is defined as the amount that releases 1 μmole of *N*-acetylneuraminic acid per min under the described reaction condition. Specific activities are expressed as units/mg protein. Protein was determined by the method of Lowry et al. [36] with bovine albumin as a standard.

Microorganisms	Colominic acid medium			Todd-Hewitt broth		
	Activity (units/ml)	Protein (μg/ml)	Spec. act.	Activity (units/ml)	Protein (μg/ml)	Spec. act.
Str. olivaceus IFO 3409	0.0426	148	0.288	0.0043	480	0.009
Streptomyces sp. MB 53-2	0.2012	155	1.298	0.0508	385	0.132
Sp. schenckii IFO 5984	0.1981	90	2.201	0.0276	354	0.078
P. urticae IFO 7011	0.0730	112	0.652	0.0089	405	0.022
Bacterium 3831-1	0.1198	120	0.998	0.0086	662	0.013

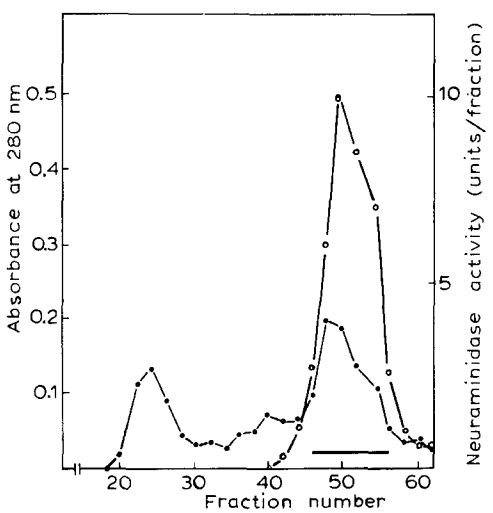

Fig. 1. Column chromatography of *Streptomyces* sp. MB 53-2 neuraminidase. Culture supernatant (500 ml) was concentrated by ultrafiltration with a collodion sac and crude enzyme preparation was salted out with 80% $(NH_4)_2SO_4$. The dialyzed 80% $(NH_4)_2SO_4$ pellet was used for the starting material for column chromatography. Samples were assayed for the enzyme activity (○—○), and the absorbance at 280 nm was measured (●—●). The assay procedures were the same as described in Table II. The active fractions indicated by the bar were pooled and concentrated by ultrafiltration with a collodion sac. Gel filtration on Sephadex G-200: The sample was applied on a column of Sephadex G-200 (2.6 cm × 100 cm) previously equilibrated with 0.025 M phosphate buffer (pH 6.8) containing 0.1 M NaCl, and eluted with the same buffer. The flow rate was 12.8 ml/h and the eluates were collected in 8.6-ml fractions.

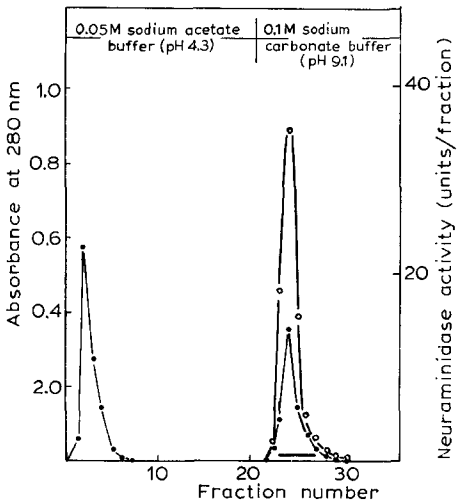

Fig. 2. Affinity chromatography. The affinity column with a selective adsorbent for neuraminidase was prepared by attaching through azo linkage *N*-(*p*-aminophenyl)oxamic acid to agarose beads containing the tripeptide, glycylglycyltyrosine [37, 38]. The concentrated active fraction from Fig. 1 was dialyzed for 15 h at 5 °C against 5 l of 0.05 M sodium acetate buffer (pH 4.3) containing 2 mM $CaCl_2$ and 0.2 mM EDTA. This material was applied to a 1.6 cm × 15 cm affinity column which had been equilibrated with the same buffer, and washed with the buffer until the absorbance at 280 nm was negligible. Elution was achieved with 0.1 M sodium carbonate buffer (pH 9.1). Eluates (5.0-ml fractions) were collected in tubes containing 0.5 ml of 0.5 M sodium acetate buffer (pH 5.0).

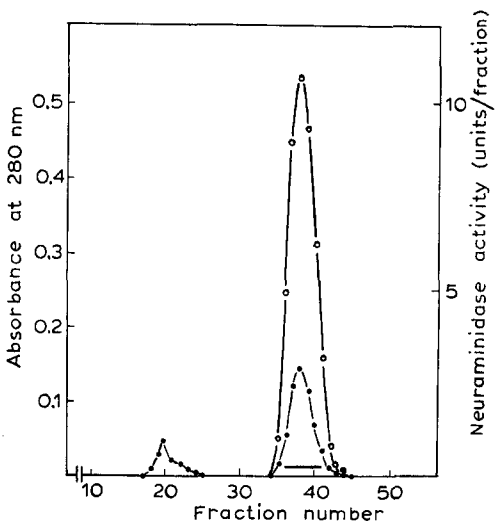

Fig. 3. Gel filtration on Sephadex G-100. The concentrated active fraction from Fig. 2 was applied on a column of Sephadex G-100 (2.6 cm × 100 cm). Chromatographic conditions were the same as shown in Fig. 1.

trichum schenckii, *Penicillium urticae*, and unidentified bacterium B 3831-1 showed potent activities. *Sp. schenckii* is a pathogenic fungus which causes sporotrichosis. The finding of strong neuraminidase activity in *Sp. schenckii* seems to be important from the medical view point.

Although these microorganisms produced neuraminidases in the culture medium such as Todd–Hewitt broth and brain heart infusion, which are usually used for the neuraminidase production, the enzyme activities were very weak compared with those obtained from the culture media adopted in this experiment (Table II).

The neuraminidases produced by these microorganisms were then purified by salting-out with $(NH_4)_2SO_4$, gel filtration, and the affinity chromatography described by Cuatrecasas and Illiano [37, 38] (Figs 1–3). A summary of the purification procedure is shown in Table III. By these steps of purification, both *Streptomyces* sp. MB

TABLE III

PURIFICATION OF NEURAMINIDASES FROM MICROORGANISMS

Culture supernatant (500 ml) was used for the preparation of neuraminidase. Assay procedure and activity definition are the same as described in Table II. For the experimental details refer to the data under *Streptomyces* sp. MB 53-2 shown in Figs 1–3. Neuraminidases from *Sp. schenckii* and B 3831-1 were purified by the same procedure adopted for *Streptomyces* sp. MB 53-2 neuraminidase except for the omission of gel filtration on Sephadex G-200.

Fraction	Protein (mg)	Spec. act.	Total activity	Purification	Yield (%)
Streptomyces sp. MB 53-2					
Culture supernatant	74.4	1.58	117.55	1.0	100
$(NH_4)_2SO_4$ (0–80%)	16.3	6.64	108.23	4.2	92.1
Sephadex G-200	9.5	11.37	108.02	7.2	91.9
Affinity chromatog.	4.4	21.49	94.56	13.6	80.4
Sephadex G-100	3.6	24.86	89.50	15.7	76.1
Sp. schenckii					
Culture supernatant	58.9	2.87	169.04	1.0	100
$(NH_4)_2SO_4$ (0–80%)	24.8	8.04	199.39	2.8	118.0
Affinity chromatog.*	6.5	21.92	142.48	7.6	84.3
Sephadex G-100	4.7	25.02	117.59	8.7	69.6
B 3831-1					
Culture supernatant	64.7	1.11	71.82	1.0	100
$(NH_4)_2SO_4$ (0–80%)	20.0	3.41	68.20	3.1	95.0
Affinity chromatog.	4.5	9.58	43.11	8.6	60.0
Sephadex G-100	4.2	10.20	42.84	9.2	59.6

* The sodium acetate buffer (pH 4.3) used in Fig. 2 is not suitable for the affinity chromatography of *Sp. schenckii* neuraminidase. At this pH the enzyme emerged without changing the buffer to sodium carbonate (pH 9.1). For the selective adsorption of this enzyme by the affinity column, sodium acetate buffer (pH 3.7) had to be used instead of the buffer (pH 4.3).

53-2 and *Sp. schenckii* neuraminidases gave a single protein band by disc electrophoresis, whereas B 3831-1 neuraminidase was separated into four bands with activities.

The purified neuraminidases are able to liberate *N*-acetylneuraminic acid not only from colominic acid in which *N*-acetylneuraminic acid is α, 2–8 linked [39, 40], but also from submaxillary mucin, ganglioside, or *N*-acetylneuramin-lactose in which *N*-acetylneuraminic acid is α, 2–6 or α, 2–3 linked (Table IV).

TABLE IV

SUBSTRATE SPECIFICITY STUDIES WITH NEURAMINIDASES FROM MICROORGANISMS

The assay procedure is the same as described in Table II. The concentration of substrates: colominic acid, 4.0 mg/ml; *N*-acetylneuramin-lactose, 4.0 mg/ml; bovine submaxillary mucin (Sigma Type I), 20.0 mg/ml; bovine brain ganglioside (Sigma Type III), 10.0 mg/ml.

Substrate	Linkage of *N*-acetylneuraminic acid	*N*-Acetylneuraminic acid released (μmoles/mg enzyme/min)		
		Streptomyces sp. MB 53-2	*Sp. schenckii*	B 3831-1
Colominic acid	α, 2–8	25.40	26.23	10.84
N-Acetylneuraminlactose	α, 2–3*	46.67	47.24	24.90
N-Acetylneuraminlactose	α, 2–6**	43.30	61.15	26.11
Bovine submaxillary mucin	α, 2–6***	11.91	9.24†	21.43
Bovine brain ganglioside	α, 2–3***	15.66	11.36	20.20

* Sigma Type II, contains approx. 15% of α, 2–6 isomer.

** Provided by Dr M. Koseki.

*** Other linkage(s) and sialic acid(s) other than *N*-acetylneuraminic acid may be contained.

† Substrate was coagulated owing to the low pH of the reaction mixture, which seems to have given a low value.

All these results show that neuraminidases with wide specificity and with strong activity can be prepared with convenience and efficiency by using a liquid medium containing colominic acid as a source of carbon. Detailed studies on the respective neuraminidase are in progress and the results will be reported elsewhere.

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