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Membrane-Bound Nucleotidase of *Bacillus cereus*†

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A membrane-bound nucleotidase of *Bacillus cereus* T was solubilized by digestion with trypsin and subsequently purified more than 300-fold. The purified nucleotidase was most active on ribonucleoside 5'-monophosphates and was slightly less active (40 to 60%) on deoxyribonucleoside 5'-monophosphates and ribonucleoside 3'-monophosphates. In addition to hydrolytic activity, the nucleotidase preparation possessed phosphotransferase activity by which phosphate is transferred from a phosphate donor to the 5' position of nucleosides.

The enzymes known collectively as nucleotidases possess a variety of specificities. The cellular location of several bacterial nucleotidases has been investigated. The 5'-nucleotidases of the *Enterobacteriaceae* and bacilli are active both as nucleoside 5'-monophosphate phosphohydrolases and as nucleoside diphosphate sugar hydrolases (11, 12). These enzymes appear to be of periplasmic origin (12, 14) and participate in the cellular uptake of nucleotides (16). The 3'-nucleotidases of the *Enterobacteriaceae* are nucleoside 3'-monophosphate phosphohydrolases and cyclic phosphodiesterases (1, 2, 13), at least some of which are also of periplasmic origin (14). The 5'-nucleotidase isolated from the growth medium of *Micrococcus sodonensis* has both monoesterase and diesterase activities (3). Cercignani et al. (6) reported the release of a 5'-nucleotidase that also possessed nucleoside diphosphate sugar hydrolase activity from intact cells of *Bacillus cereus* by washing the cells with water, indicating that this enzyme is located on the cell surface. An enzyme purified from extracts of *Escherichia coli* possesses nucleotide phosphotransferase activity that transfers organically esterified phosphate from suitable donors to nucleosides, yielding the corresponding 2'- and 3'-nucleotides (4, 5). In the absence of an acceptor, the phosphate donor is hydrolyzed by the phosphotransferase in the fashion of 2'- or 3'-nucleotidase. The nucleoside phosphotransferase of *Erwinia herbicola* is a membrane-bound enzyme that can utilize both 3'- and 5'-nucleotides as phosphate donors (7). This enzyme has very little activity as a nucleotide phosphohydrolase.

In this paper, we report a membrane-bound nucleotidase of *B. cereus* T that we have solubilized from the membrane and subsequently purified and partially characterized. In contrast to the combinations of specificities noted above, this nucleotidase possesses 5'-nucleotidase, 3'-nucleotidase, and phosphotransferase activities but does not possess appreciable nucleoside diphosphate sugar hydrolase or cyclic phosphodiesterase activities.

MATERIALS AND METHODS

Growth medium. One liter of growth medium contained 2 g of yeast extract, 2 g of peptone, 0.5 g of K_2HPO_4, 50 ml of a solution of glucose (20 g/liter), and 10 ml of a solution of inorganic salts. The inorganic salts solution contained 0.10 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of $CuSO_4 \cdot 5H_2O$, 1.0 g of $ZnSO_4 \cdot 7H_2O$, 10 g of $MnSO_4 \cdot H_2O$, 40 g of $MgSO_4$, and 200 g of $(NH_4)_2SO_4$ dissolved in 1 liter of water. The glucose solution and the inorganic salts solution were autoclaved separately from the remainder of the medium.

Growth of *B. cereus*. A preparation of spores of *B. cereus* T was kindly provided by the late H. O. Halvorson, then of the Department of Biochemistry, University of Minnesota. Approximately 5 μl of *B. cereus* T spores (10^7 spores per μl) was used to inoculate 100 ml of growth medium. After 10 h of incubation with shaking at 30°C, 5-ml portions of the culture were used to inoculate 1-liter batches of growth medium. Incubation was continued with vigorous shaking at 30°C until the cultures reached mid-log phase as determined by turbidity measurements at 700 nm. The cultures were cooled rapidly in ice and harvested by centrifugation at 4°C. The cells were washed twice in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, and the wet-packed cells (usually about 1.5 g/liter of medium) were used immediately for enzyme preparation.

Sporulation of *B. cereus* T was achieved by incubation in growth medium supplemented with $CaCl_2$ (0.08 g/liter). After 16 to 20 h of incubation with vigorous shaking at 30°C, the spores were collected by centrifugation, washed twice with sterile water, resuspended in sterile water, and stored at −10°C.

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Isolation of nucleotidase. Step I. A 20-g amount of wet-packed cells and 10 g of 75-μm glass beads were suspended in 100 ml of 0.1 M Tris-hydrochloride, pH 8.0, and disrupted by sonic treatment in an ice-water bath with a Heat Systems Ultrasonics sonic oscillator until the cell disruption appeared complete by microscopic examination.

Step II. After removal of the glass beads, 1 ml of Nonidet P-40 and 1 mg each of deoxyribonuclease and ribonuclease were added. After stirring for 24 h at 4°C, the suspension was warmed to room temperature, the pH was adjusted to 8.0 with 1 M Tris base, and 10 mg of trypsin was added. After 5 h of incubation at room temperature, an additional 10 mg of trypsin was added. The mixture was stored overnight at 4°C, after which the suspension was centrifuged at 48,000 × *g* for 30 min to remove particulate matter.

Step III. The supernatant solution was mixed with 3 volumes of cold acetone, allowed to stand for 45 min at 0°C, and centrifuged at 48,000 × *g* for 30 min. The precipitate was extracted twice with 15-ml volumes of 1.0 M ammonium acetate, pH 5.0, containing 1% Nonidet P-40 and centrifuged each time at 48,000 × *g* for 30 min. The combined extracts were precipitated as before with acetone. The acetone precipitate was dissolved in a minimum volume (2 ml) of 0.1 M Tris-hydrochloride, pH 8.0.

Step IV. The solution of nucleotidase was applied to a column of Sephadex G-100 (1.8 by 100 cm) previously equilibrated with 0.1 M Tris-hydrochloride, pH 8.0. The column was developed with the same buffer at a flow rate of 6 ml/h. The fractions containing nucleotidase activity were pooled and dialyzed overnight against 2 liters of distilled water.

Step V. The dialyzed nucleotidase from the Sephadex G-100 column was electrofocused in an LKB electrofocusing apparatus in a pH 5 to 8 gradient. The fractions that electrofocused at pH 6.0, 6.8, 6.9, and 7.1 were dialyzed separately and exhaustively against 0.1 M Tris-hydrochloride, pH 8.0. The purified nucleotidase was stored at 4°C.

The concentration of protein was determined by the Lowry procedure (10), using bovine serum albumin as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Davis (9) at room temperature in 0.5 M Tris-hydrochloride buffer, pH 8.9. Samples weighted with sucrose were layered directly on the sieving gel. The gels were stained for protein with Coomassie brilliant blue. Fractionation of gels was accomplished with an extruder.

Nucleotidase activity was located on polyacrylamide gels by an adaptation of a histological technique (15). After electrophoresis, the gels were washed in 1.0 M Tris-acetate, pH 7.0, for 15 min and then placed in a solution of the same buffer containing 2.5 mM nucleoside monophosphate, 2.5 mM magnesium acetate, and 1.0 mM lead(II) nitrate. During incubation at room temperature, the inorganic phosphate produced by the enzymatic hydrolysis of the nucleotide was deposited as a ring of lead phosphate on the surface of the gel. The reaction was stopped by immersing the gels in boiling water for 3 min. The definition of the bands faded slowly over a period of several days.

Nucleotidase assays. Nucleotidase activity was assayed at pH 5.6 and also at pH 8.2. The assay mixture consisted of 2.5 mM substrate (5′-UMP unless otherwise indicated), 2.5 mM $MgCl_2$, and 0.25 M ammonium acetate (pH 5.6) together with an appropriate amount of the nucleotidase preparation (0.06 μg of protein of the most purified preparation) in a total volume of 0.1 ml. For determinations at pH 8.2, the assay mixture contained 0.25 M Tris-hydrochloride, pH 8.2, as a substitute for the ammonium acetate. The amount of inorganic phosphate liberated during incubation at 37°C was determined by the method of Chen et al. (8).

Phosphotransferase assays. To determine phosphotransferase activity, reaction mixtures as described for nucleotidase assays were supplemented with 2.0 mM [^{14}C]uridine. During incubation at 37°C, portions were withdrawn at intervals and immediately inactivated by addition to an equal volume of isobutyric acid. The inactivated portions were spotted on Whatman 3MM filter paper and subjected to descending chromatography in isobutyric acid–1 M NH_4OH (5:3, vol/vol). After chromatography, the radioactive spots of uridine and 5′-UMP, located by the absorption of UV light by the corresponding reference compounds, were cut out and placed in scintillation vials with scintillation fluid (4 g of diphenyloxazole per liter of toluene) and counted. The percent distribution of the radioactivity was used to quantitate the activity of the phosphotransferase.

RESULTS

Nucleotidase activity. Particulate enzyme preparations, which include fragments of the cell membrane obtained from cells of *B. cereus* T disrupted by sonic oscillation or by grinding with alumina, contained an enzymatic activity that rapidly converted 5′-UMP to uridine and orthophosphate. From 5′-[^{14}C]UMP, the enzymatic activity produced a single radioactive compound with the paper chromatographic mobility of uridine. Orthophosphate was identified as a reaction product by colorimetric determination. The identification of reaction products, together with the results of the specificity studies described below, supports the designation of this enzymatic activity as a nucleotidase.

Solubilization of nucleotidase. To verify that the nucleotidase was initially membrane bound, the cell extract obtained by grinding cells with alumina was separated by centrifugation for 2 h at 80,000 × *g* into a cell envelope fraction containing both cell wall and cell membrane fragments and a supernatant fraction containing the soluble cytoplasmic constituents. Determination of the amount of nucleotidase activity in these fractions showed that 98% of the total activity was recovered with the fraction that included the cell membrane fragments. Washing of this fraction by resuspension in water and resedimentation did not release significant quan-

tities of nucleotidase from the particulate fraction.

To evaluate the solubilization of the membrane-bound enzyme, preparations containing the nucleotidase were applied to 4% polyacrylamide gels and subjected to electrophoresis. The mobility of the nucleotidase activity was determined either by elution from gel sections followed by quantitative assay in vitro or by a modification of a histological technique in which lead phosphate is deposited in those portions of the gel containing the nucleotidase. The nucleotidase was considered to have been solubilized if the enzymatic activity penetrated the polyacrylamide gel and migrated as a distinct component.

Most of the nucleotidase associated with cell membrane fractions did not penetrate polyacrylamide gels upon electrophoresis but remained at the surface of the gel. When a crude cell envelope fraction was stirred with 1% Nonidet P-40 (a nonionic detergent) for 24 h at 4°C, 90% of the nucleotidase activity could no longer be sedimented by centrifugation for 2 h at 80,000 × *g*. However, when the supernatant fraction containing the nucleotidase partially solubilized by the detergent was subjected to electrophoresis, most of the nucleotidase activity was excluded from the gel, and that which penetrated was smeared over the upper portion of the gel. A number of other approaches for solubilizing membrane-bound enzymes were also tried. Treatments with several different concentrations of KCl at high and low pH, deoxycholate, urea, or guanidine hydrochloride, and various combinations of these solubilizing agents were tried without success. It was found, however, that after a portion of the Nonidet P-40-treated nucleotidase had been incubated with trypsin for 3 h at room temperature, the nucleotidase activity penetrated the gel and migrated as a single diffuse band. On the basis of these observations, the procedure developed for release of the enzyme from the membranes used the partial solubilization by Nonidet P-40 followed by incubation with trypsin. With this combination of treatments, essentially all (98%) of the nucleotidase activity entered the gel upon electrophoresis.

Purification of solubilized nucleotidase. Table 1 presents a summary of the data obtained by the use of the solubilization and purification procedure adopted. The complete recovery of units of nucleotidase activity after treatment with trypsin indicates that the activity of the nucleotidase is not destroyed by trypsin. The nucleotidase was found to be resistant to inactivation by trypsin at all stages of the purification. Consequently, no special measures were employed to inactivate or selectively remove the trypsin after solubilization of the nucleotidase had been effected.

The substrate specificity of the hydrolytic activity found in membrane preparations suggested that perhaps more than one nucleotidase might be present in the crude enzyme preparation. Consequently, the ratio of hydrolytic activity toward 3′-UMP to that toward 5′-UMP was determined at all stages of purification. The ratio of activity toward these substrates after step I was the same as that for maximally purified enzyme. Therefore, the loss of activity observed with the gel filtration step is probably not due to the removal or inactivation of other nucleotidases. The equilibration of the gel filtration column with detergent and the use of a developing buffer containing detergent did not significantly improve recovery. Preparations of the nucleotidase recovered from the gel filtration columns readily adsorbed to glass. The addition of bovine serum albumin to a concentration of 0.025 mg/ml in nucleotidase assay mixtures eliminated irregularities in assay results caused by adsorption.

The activity profile of the enzyme electrofocused over a pH 5 to 8 gradient (step V) is shown in Fig. 1. Peaks of nucleotidase activity electrofocused at pH 6.0 and at pH 6.9. The ratio of hydrolysis of 3′-UMP to 5′-UMP at pH 5.6 and the ratio of hydrolysis of 5′-UMP at pH 5.6 to that at pH 8.2 (see below) were constant over the gradient and were the same as the ratios observed with the preparation applied to the electrofocusing column. Portions of the nucleotidase activity peaks subjected to electrophoresis on polyacrylamide gels gave similar lead phosphate precipitation patterns when incubated with 5′-UMP and lead nitrate (see be-

TABLE 1. *Summary of the purification of the nucleotidase*

	Purification step	Total activity (μmol/min)	Recovery (%)	Sp act (μmol/min per mg of protein)	Purification	$V_{(3'\text{-UMP})}/V_{(5'\text{-UMP})}$
I	Crude extract	873	100	0.14		0.45
II	Trypsin treatment	880	101	0.17	1.2	0.37
III	Acetone precipitate	463	53	2.4	19	0.46
IV	Sephadex G-100	37.6	4.3	7.1	51	0.45
V	Electrofocusing	23.7	2.7	46	328	0.49

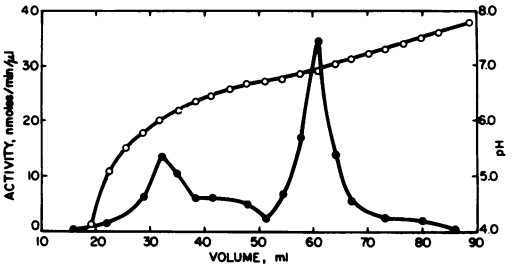

FIG. 1. *Electrofocusing of nucleotidase. Nucleotidase from step IV was electrofocused in a pH 5 to 8 gradient at 10°C. The hydrolytic activity was assayed at pH 5.6 with 5'-UMP as the substrate. Symbols: ●, nucleotidase activity; ○, pH.*

low). The three most active fractions of the activity peak that electrofocused at pH 6.9 were purified more than 300-fold with respect to the crude extract. The most active fraction was used as the source of purified enzyme for all subsequent studies unless otherwise stated. The purified nucleotidase preparation retained approximately 90% of the original hydrolytic activity after 6 months of storage at 4°C. The ratio of activity toward 3'-UMP and 5'-UMP remained constant during storage, as did the ratio of hydrolysis of 5'-UMP at pH 5.6 to that at pH 8.2.

Optimum reaction conditions. The nucleotidase was active between pH 4.5 and 9.0. Maximum activity was observed at pH 5.6 for hydrolysis of 5'-UMP and 3'-UMP. At pH 8.2 the activity toward 5'-UMP was 78% of that at pH 5.6. When the substrate was 3'-UMP, the activity at pH 8.2 was only 10% of that at pH 5.6. The pH-activity plots were essentially identical for preparations of membrane-bound nucleotidase that had not been treated with trypsin.

The maximum activity of the nucleotidase was observed in the presence of magnesium ions. The effect of magnesium ions was independent of the concentration over the range of 0.1 to 10 mM magnesium ions. Pretreatment of the nucleotidase with 0.45 mM ethylenediaminetetraacetic acid reduced the activity observed in the presence of 1.0 mM magnesium ions by 30% and accentuated the dependence upon divalent cations. Cobalt(II) and zinc(II) ions at 1.0 mM could substitute for magnesium ions, giving activities 85 to 95% of those with magnesium ions. Manganese ions at 1.0 mM gave an activity only 30% of that with magnesium ions. Calcium ions were inhibitory. Although these results do not indicate an absolute requirement for magnesium ions, the increased activity observed in their presence prompted the inclusion of 2.5 mM magnesium ions in all routine assay mixtures for the nucleotidase.

The nucleotidase had a K_m value for 5'-UMP of 0.007 mM and for 3'-UMP of 0.2 mM. Thus, the nucleotidase assayed in mixtures that contained either 5'-UMP or 3'-UMP at a concentration of 2.5 mM was saturated with respect to substrate.

Purity of nucleotidase. When the most purified fraction of nucleotidase was subjected to electrophoresis on 10% polyacrylamide gels at pH 8.9 and subsequently stained for protein with Coomassie brilliant blue, a pair of bands having nearly identical mobilities was observed (Fig. 2). Both bands were shown to contain nucleotidase activity by the deposition of lead phosphate in situ with either 5'-UMP or 3'-UMP as the substrate. Nucleotidase activity was also demonstrated in each band by fractionation of the gel into 1-mm sections followed by quantitative assays in vitro. The specific activities of the two bands were approximately equal. No other portions of the gels contained nucleotidase activity. There was no difference between the two bands with respect to the ratio of rates of hydrolysis of 5'-UMP and 3'-UMP. The pair of enzymatically active bands was observed at all stages of purification of the nucleotidase subsequent to treatment with trypsin. Additional exposure of the most purified fraction of nucleotidase to trypsin did not alter the mobilities of the two bands of nucleotidases, their relative proportion, or their ability to hydrolyze both 5'-UMP and 3'-UMP.

Because in some bacteria the 5'- and 3'-nucleotidases are separate and distinct enzymes, an attempt was made to demonstrate a differential heat inactivation of the activities that hydrolyze 5'-UMP and 3'-UMP. Portions of the purified nucleotidase were incubated for 10 min in 0.1 M Tris-hydrochloride, pH 8.2, at temperatures in the range from 30 to 80°C and subsequently assayed for hydrolytic activity toward 5'-UMP and 3'-UMP. There was no significant difference in the heat inactivation of the two activities (Fig. 3). Similar results were obtained when the heat inactivation was conducted in 0.1 M ammonium acetate, pH 5.6.

Treatments with trypsin and Pronase were also examined in the search for differential effects on the two hydrolytic activities. Figure 4 shows that both activities were essentially unaffected by incubation with trypsin for 130 min. In contrast, digestion with Pronase caused a loss of both activities at a nearly uniform rate for 20 min, after which the activity for hydrolysis of 3'-UMP was more rapidly destroyed than that for hydrolysis of 5'-UMP.

Molecular weight. A preparation of acetone-precipitated nucleotidase (step III) was applied to a Sephadex G-100 column that had been calibrated with several globular proteins of

FIG. 2. *Polyacrylamide gel electrophoresis of nucleotidase. Nucleotidase (0.01 mg) from step V was subjected to electrophoresis on 10% polyacrylamide gels at pH 8.9. The gel was stained with Coomassie brilliant blue.*

known molecular weight. The nucleotidase activity eluted as a single sharp peak at an elution volume between that observed for ovalbumin (4.35×10^4 daltons) and carbonic anhydrase

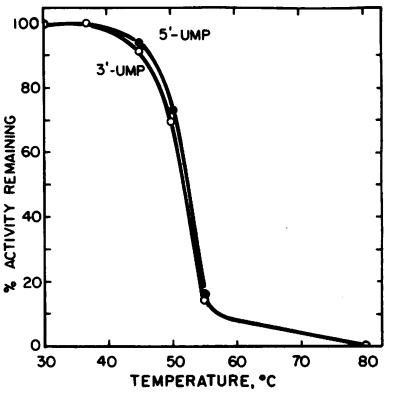

FIG. 3. *Heat inactivation of nucleotidase activities. Nucleotidase was incubated for 10 min at the temperature indicated in 0.1 M Tris-hydrochloride, pH 8.0. To determine residual enzymatic activity, portions of the heated nucleotidase were added to 5 volumes of 1.0 M ammonium acetate, pH 5.6, containing 0.1 mg of bovine serum albumin per ml. Substrates used for the assay of nucleotidase activities were 5'-UMP (●) and 3'-UMP (○).*

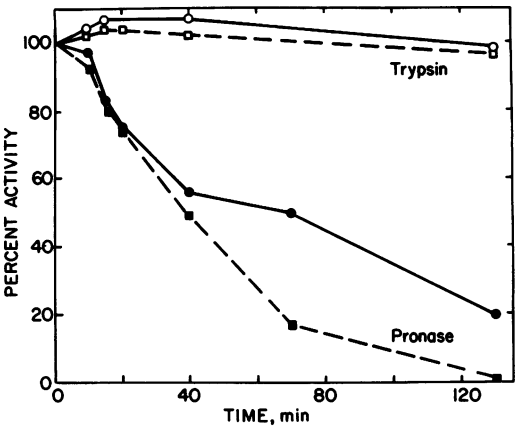

FIG. 4. *Inactivation by proteolytic enzymes. Nucleotidase (0.2 mg/ml) was incubated at 27°C with trypsin (0.5 mg/ml, open symbols) or with Pronase (0.055 mg/ml, closed symbols) in 0.1 M Tris-hydrochloride, pH 8.0. At the times indicated, portions were withdrawn and diluted threefold with 1.0 M ammonium acetate, pH 5.6, containing 0.05 mg of bovine serum albumin per ml. $MgCl_2$ and 5'-UMP (○, ●) or 3'-UMP (□, ■) were added to initiate the assay for nucleotidase activity. Nucleotidase assay mixtures were incubated for 10 min.*

(3.06×10^4 daltons). Assuming that the nucleotidase behaves as a globular protein, its molecular weight as determined from a plot of log molecular weight versus K_{av} is approximately 3.5×10^4.

Substrate specificity. The relative activities of the purified nucleotidase toward a number of nucleotides and other possible substrates at pH 5.6 and also at pH 8.2 are given in Table 2. The activities of the nucleotidase not exposed to trypsin are also listed in Table 2. It appears that trypsinization did not significantly alter the substrate specificity of the nucleotidase. The ribonucleoside 5'-monophosphates were hydrolyzed most rapidly. The deoxyribonucleoside 5'-monophosphates and the ribonucleoside 3'-monophosphates were hydrolyzed at 40 to 60% of the rate of the 5'-ribonucleotides. The position of the phosphate moiety in the nucleotide was of greater influence on the rate of hydrolysis than was the purine or pyrimidine base. The enzyme showed little hydrolytic activity toward other phosphomonoesters with the exception of ribose-5-phosphate and *p*-nitrophenylphosphate. There was neither detectable release of inorganic phosphate from nucleoside 2',3'- or 3',5'-cyclic monophosphates nor any detectable cyclic phosphodiesterase activity. When nucleotidase assay mixtures containing nucleoside cyclic monophosphates were subjected to paper chromatography in isobutyric acid–1 M NH_4OH (5:3, vol/vol), all of the UV-absorbing material remained as the nucleoside cyclic monophosphates; no nucleoside monophosphates could be detected. The rates of hydrolysis of UDP and UTP were 12 and 0.3% respectively, of that of 5'-UMP at pH 5.6. [^{14}C]UDP was hydrolyzed to uridine with no detectable accumulation of

TABLE 2. *Substrate specificity of nucleotidase*

Substrate[b]	Relative activity[a]			Substrate[b]	Relative activity[a]		
	Nucleotidase not exposed to trypsin[c] (pH 5.6)	Purified nucleotidase			Nucleotidase not exposed to trypsin[c] (pH 5.6)	Purified nucleotidase	
		pH 5.6	pH 8.2			pH 5.6	pH 8.2
5'-UMP	100	100	78	3',5'-UMP	<0.1	<0.1	<0.1
5'-CMP	—[d]	102	76	2',3'-AMP	—	<0.1	<0.1
5'-AMP	—	75	62	3',5'-AMP	<0.1	<0.1	<0.1
5'-GMP	112	100	96				
				UDP[e]	11	12	0.5
5'-dUMP	46	45	45	UTP[e]	1	0.3	0.1
5'-dCMP	—	67	55				
5'-dTMP	—	55	40				
5'-dAMP	—	50	36	NADP[f]	—	7	1
5'-dGMP	57	48	65	UpGp	—	7	—
				Polyuridylic acid	—	<1[g]	<1[g]
3'-UMP	45	49	5	UDP-glucose	—	<1[g]	—
3'-CMP	—	32	2	UDP-GlcNAc	—	0.1	—
3'-AMP	—	68	15				
3'-GMP	64	60	9	Ribose-5-phosphate	20	38	—
				Glucose-6-phosphate	1	1	0.1
2'-AMP	8	11	—	α-Glycerophosphate	—	14	<0.1
				p-Nitrophenylphosphate	114	113	16
2',3'-UMP	<0.1	<0.1	<0.1				

[a] Activity is expressed relative to the rate of hydrolysis of 5'-UMP at pH 5.6, which is arbitrarily assigned a value of 100. The specific activity of the purified nucleotidase was 13 μmol/min per mg of protein.

[b] Substrate concentrations were 2.5 mM.

[c] Nucleotidase not exposed to trypsin was prepared from the crude extract (step I) by centrifugation at 100,000 × *g*, resuspension of the pellet in 1% Nonidet P-40 at pH 8.0 (24 h at 4°C), removal of material not solubilized by centrifugation at 40,000 × *g*, and isoelectric precipitation of the nucleotidase between pH 5.0 and 4.2. The precipitate recovered by centrifugation was dissolved in 0.1 M Tris-hydrochloride, pH 8.0, containing 1% Nonidet P-40.

[d] Not determined.

[e] Because measurement of the rate of hydrolysis is dependent upon the rate of release of inorganic phosphate, the relative rates reported here for UDP and UTP have been calculated from the amount of inorganic phosphate released, assuming that each 1 mol of UDP hydrolyzed yields 2 mol of inorganic phosphate and that each 1 mol of UTP hydrolyzed yields 3 mol. These results were confirmed with [^{14}C]UDP and [^{14}C]UTP.

[f] NADP, Nicotinamide adenine dinucleotide phosphate.

[g] The high background color yield of these compounds in the assay for inorganic phosphate precluded more accurate determinations of activity.

UMP, as determined by radioactivity measurements after paper chromatography of reaction mixtures in isobutyric acid–1 M NH_4OH (5:3, vol/vol). [^{14}C]UTP was hydrolyzed to uridine without the formation of detectable amounts of UDP or UMP. At pH 8.2, the nucleotidase was less active than at pH 5.6, although the decrease was relatively smaller from the ribonucleoside 5′-monophosphates and deoxyribonucleoside 5′-monophosphates than for the other nucleotides and phosphomonoesters.

Phosphotransferase activity. In addition to hydrolytic activity, the nucleotidase was found to possess phosphotransferase activity. When [^{14}C]uridine was incubated with 5′-UMP and the nucleotidase, 5′-[^{14}C]UMP was produced. Several compounds were examined for their ability to function as phosphate donors (Fig. 5). The most effective donors were the nucleoside 5′-monophosphates. Phosphotransferase activity was greater at pH 8.2 than at pH 5.6. 5′-UMP was an effective phosphate donor at both pH values, whereas 3′-UMP was only marginally effective as a phosphate donor. The product of the phosphate transfer from 5′-UMP or 3′-UMP to [^{14}C]uridine at either pH was identified as 5′-[^{14}C]UMP by paper chromatographic separation of 5′-UMP from 3′-UMP in isobutyric acid–1 M NH_4OH (5:3, vol/vol).

After the nucleotidase preparation had been subjected to polyacrylamide gel electrophoresis, segments of the gel were assayed for nucleotidase activity and phosphotransferase activity. Both activities were observed in the same region of the gel, suggesting that both enzyme activities are expressions of the same protein.

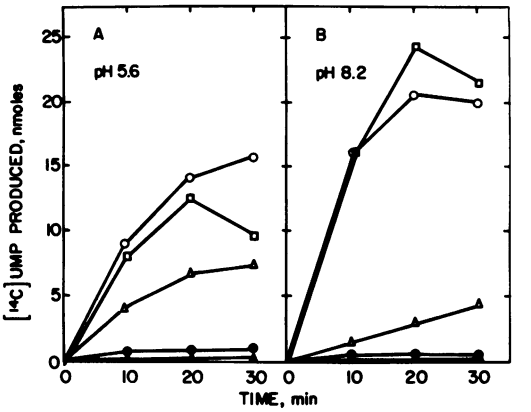

FIG. 5. *Phosphotransferase activity of nucleotidase. The nucleotidase preparation (step V) was incubated with 50 nmol of [^{14}C]uridine and the indicated phosphate donors at pH 5.6 (A) and at pH 8.2 (B). Details of the phosphotransferase assay are given in the text. Phosphate donors were 5′-UMP* (○), *5′-AMP* (□), *p-nitrophenylphosphate* (△), *3′-UMP* (●), *UDP or UTP* (▲).

DISCUSSION

The results presented above show that the nucleotidase that has been solubilized from a particulate fraction of *B. cereus* has rather broad specificity in that it readily hydrolyzes 5′-nucleotides and 3′-nucleotides and also displays phosphotransferase activity. Although in some bacteria the 5′-nucleotidases are enzymes distinctly different from the 3′-nucleotidases (1–3, 11–13), the evidence presented here suggests that in *B. cereus* the same protein is responsible for both activities. The observations that the ratio of hydrolytic activity toward 3′-UMP relative to that toward 5′-UMP did not change significantly during purification, that both activities migrated identically when the purified nucleotidase was subjected to polyacrylamide gel electrophoresis, and that both activities displayed the same sensitivity to inactivation by heat suggest that a single enzyme is involved. The observation of a more rapid loss of hydrolytic activity toward 3′-UMP compared with that toward 5′-UMP after treatment with Pronase provided the only experimental evidence not consistent with this suggestion. One interpretation of this observation is that there are two separate enzymes that are differentially inactivated by the action of Pronase. Alternatively, because the assays for nucleotidase activities employ two different substrates with substantially different K_m values, it is possible that proteolysis results in an accentuation of the differing affinity of a single enzyme for these substrates. Based on our results, the possibility that the two nucleotidase activities might be due to different enzymes cannot be excluded.

The most purified preparation of the nucleotidase was resolved into the major protein components by polyacrylamide gel electrophoresis. Both of the components were found to be enzymatically active. Other than the slightly dissimilar electrophoretic mobilities, no detectable differences between the two bands were found. Because this doublet was seen after release from membrane fragments by trypsin, it is possible that these two proteins represent the products of proteolytic cleavage at the different points on a common polypeptide chain. The fact that both components persist even with prolonged digestion with trypsin suggests that cleavage at one point may preclude subsequent hydrolytic action at the other or that one of the cleavage points may result from the action of an intrinsic protease. Alternatively, the difference in the electrophoretic mobility of the proteins might be due to the difference of a charged group at a position not crucial to the specificity of the en-

zyme or be due to a variation in lipid or carbohydrate content of two otherwise identical polypeptide chains.

Because solubilization of the nucleotidase was later achieved by incubation of crude cell extracts with trypsin but without prior exposure to Nonidet P-40, it may be possible to eliminate the detergent treatment from the purification procedure. An unfortunate aspect of the solubilization procedure that was employed is that the digestion with trypsin may have caused some subtle changes in the specificity of the solubilized form of the enzyme. However, examination of a partially purified preparation of nucleotidase that had been treated with detergent but that had not been exposed to trypsin did not display any significant differences from the specificity reported for the trypsin-solubilized nucleotidase.

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