Polymyxin Acylase: Purification and Characterization, with Special Reference to Broad Substrate Specificity

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Polymyxin acylase, which produces deacylated polymyxins by hydrolyzing only the fattyacyl groups of polymyxin antibiotics without affecting the peptide moiety, was purified from acetone-dried cell powder of *Pseudomonas* sp. M-6-3. The cell-free enzyme, solubilized by Triton X-100, was further purified on successive DEAE-cellulose, hydroxyapatite, and Sephacryl S-300 columns to a homogeneous state. This purified enzyme (Type I) had a single band with an MW of 62,000 in SDS-polyacrylamide gel electrophoresis. Gel filtration on a calibrated Sephacryl S-300 column also gave an estimated MW of 62,000. The isoelectric point of the enzyme was 5.7. Enzyme activity was optimal at pH 9.0 for colistin A (polymyxin E₁) and was stable up to 50°C for 24 hr. The observed $V_{\max}$ and K_m values were 1750 nmol/min/mg and 3.85 mM, respectively. This enzyme was almost not affected by metal chelators and various thiol-enzyme inhibitors (other than *p*-chloromercuribenzoate), and showed high tolerance for organic modifiers; for example, half of the activity remained in 50% ethylene glycol buffer. This enzyme deacylated not only polymyxins but also various *N*-fattyacyl compounds (peptides and amino acids). Among several fattyacyl groups (C₂–C₁₆) of *N*-acyl DL-methionines, the caprinoyl (C₁₀) group was most easily liberated, and the benzyloxycarbonyl (Z) group was also slightly susceptible. When the enzyme was solubilized in 0.2 M KCl-containing buffer by Triton X-100, the enzyme (Type II) showed a slightly different substrate specificity and an increased activity for some Z-derivatives. With this enzyme, it is possible to remove the Z group from several Z-peptides under mild conditions.

The acyl peptide antibiotics, polymyxin B and colistin (polymyxin E), have been used clinically against Gram-negative bacteria. However, little has been done to improve their therapeutic value by creating semisynthetic polymyxins *via* replacement of the acyl group. Previously, we found a strain, *Pseudomonas* sp. M-6-3,¹⁾ which produces polymyxin acylase, a new enzyme for preparing the starting material for semisynthetic polymyxins. We showed that the cell-bound enzyme acts on polymyxin to produce deacylated polymyxin and fatty acid(s). Furthermore, the cell-bound enzyme can produce deacylated peptides from the acyl peptide antibiotics, cerexin (by Shoji & Kato)²⁾, brevistin (by Shoji & Kato),³⁾ echinocandin B (by Pache *et al.*),⁴⁾ and neopeptin

(by Ubukata *et al.*).⁵⁾ This paper is concerned with the solubilization, purification, and characterization of polymyxin acylase from *Pseudomonas* sp. M-6-3. Since polymyxin acylase is unique in having a broader substrate specificity than several other known aminoacylases, it should be useful as a deacylating reagent for many *N*-acyl compounds.

Materials and Methods

Strain and growth conditions. *Pseudomonas* sp. M-6-3 strain was isolated in our laboratory from a soil sample from Nishinomiya, Japan.¹⁾ The organisms were grown aerobically in medium containing 10 g of sodium citrate, 0.1 g of yeast extract, 2 g of (NH₄)₂SO₄, 1 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 0.01 g of FeCl₃·6H₂O, and 0.01 g

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; TLCK, *p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride; DFP, diisopropylphosphoro fluoridate; Z, *N*-benzyloxycarbonyl.

of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter of water at pH 7.5 which was shaken at 28°C for 72 hr. The bacterial mass was harvested, and the acetone-dried cell powder was prepared by the usual method, giving a yield of 0.9 g/l.

Chemicals. Colistin sulfate was kindly supplied by the Banyu Pharmaceutical Co. (Tokyo, Japan). Colistin A was prepared from a colistin complex by HPLC and *N*-fattyacyl methionines were prepared by the water-soluble active ester method⁶⁾ from the appropriate fattyacyl ester of *p*-dimethylsulfonylphenol methylsulfate and methionine in our laboratory. *N*-Benzyloxycarbonyl peptides were the generous gifts of Dr. M. Fujino, Takeda Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan).

Enzyme assay. Polymyxin acylase was assayed from the UV-absorption peak of deacylated peptide on HPLC (method 1). Acylase activity for acyl amino acids was assayed by the ninhydrin color of the liberated amino acid (method 2). Urethane-hydrolase activity for *N*-benzyloxycarbonyl derivatives was assayed from the UV-absorption of the liberated benzyl alcohol on HPLC (method 3).

Assay method 1. The reaction mixture, 200 μl , containing 2 μmol of colistin sulfate and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 9.0), was incubated at 37°C for several hours. After each hour, 100 μl of 1 *N* H_2SO_4 was added and the mixture was filtered using a 0.45- μm filter (TOSOH, Tokyo, Japan). A 10- μl portion of the filtrate was injected into the HPLC [column, Ultrasphere ODS 5 μm (4.6 $\times$ 250 mm) (Beckman, U.S.A.); mobile phase, $\text{MeOH} : 0.01 \text{ N } \text{H}_2\text{SO}_4 = 1 : 1$; detector, UV (210 nm)]. The amount of deacyl colistin was calculated from the height of the peak on the chromatogram. One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of deacyl colistin per min.

Assay method 2. The reaction mixture, 200 μl , containing 10 μmol of acyl amino acid (usually *N*-capryloyl-DL-methionine) sodium salt, and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 8.0), was incubated at 37°C for several hours. The reaction mixture was treated as in method 1 and the amount of liberated amino acid in 100 μl of the filtrate was measured colorimetrically by the ninhydrin method.

Assay method 3. The reaction mixture, 200 μl , containing 1 μmol *N*-benzyloxycarbonyl peptide and the enzyme solution (100 μl) in 50 mM Tris buffer (pH 8.0) was treated as in method 1. Next, 10 μl of the enzyme reaction solution was injected into the HPLC [column, Shodex OHpak B-804 (8.0 $\times$ 500 mm) (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, water; detector, UV (210 nm)]. The amount of the liberated benzyl alcohol was calculated from the height of the peak on the chromatogram.

Solubilization of the enzyme from acetone-dried cell

powder. The powder prepared above (100 mg) was used throughout the following treatments.

a) Sonication: The powder was suspended in 10 ml of 100 mM phosphate buffer (pH 7.2), and disrupted in the presence of a few drops of toluene with a 10 kHz ultrasonic oscillator for 30 min. The cellular debris was removed by centrifugation at $10,000 \times g$ for 30 min.

b) EDTA-Lysozyme: The powder was suspended in 10 ml of 5 mM phosphate buffer (pH 6.8), containing 50 μmol EDTA and 50 μmol 2-mercaptoethanol, and then digested by the addition of egg white lysozyme (100 $\mu\text{g}/\text{ml}$) at 30°C for 4 hr. The broth was centrifuged to remove cellular debris.

c) Colistin: The powder was suspended in 10 ml of 10 mM phosphate buffer (pH 7.2), containing 1 mg of colistin sulfate and 1 μmol 2-mercaptoethanol. The mixture was stirred at 28°C for 15 hr, and centrifuged.

d) Triton X-100: The powder was suspended in 10 ml of 10 mM phosphate buffer (pH 7.2), containing 10 mg of Triton X-100 and 1 μmol 2-mercaptoethanol. The mixture was stirred at 28°C for 15 hr and then centrifuged.

Protein measurement. Protein was measured by the method of Lowry *et al.*,⁷⁾ with bovine serum albumin (Sigma Chemical Co., U.S.A.) as the standard. In all chromatographic procedures the protein concentration was measured by the absorbance at 280 nm.

Purification of enzyme. One gram of acetone-dried cell powder of *Pseudomonas* sp. M-6-3 was suspended in 30 ml of 10 mM phosphate buffer (pH 8.0) containing 100 mg of Triton X-100. The suspension was stirred at 20°C for 15 hr and then centrifuged at $10,000 \times g$ for 30 min. The supernatant was put on a DEAE-cellulose (DE53; Whatman, U.K.) column (1.5 $\times$ 21 cm; equilibrated with 10 mM phosphate buffer at pH 8.0), eluted first with the same buffer, and then with a linear gradient from 0 to 500 mM KCl in the equilibration buffer. The active fractions were effectively eluted in the first step. Because the capacity of the column was small, this procedure was repeated several times and the active fractions were pooled. The enzyme from the above step was put on a hydroxyapatite (Nippon Chemical Co., Japan) column (1.5 $\times$ 14 cm; equilibrated with 10 mM phosphate buffer at pH 8.0), eluted first with the same buffer, and then with a linear gradient from 10 to 500 mM of phosphate (pH 8.0). The active fractions emerged at two peaks: Fr. I, which showed the most enzyme activity, was re-chromatographed on a hydroxyapatite column as above. The resulting active fractions were combined and then concentrated with a Millipore Immersible-CX Ultrafilter. This concentrated enzyme was put on a Sephacryl S-300 (Pharmacia Fine Chemicals Co., Sweden) column (1.5 $\times$ 82 cm; equilibrated with 10 mM phosphate buffer at pH 8.0). The lower molecular weight fraction was re-chromatographed on a Sephacryl S-300 column. The enzyme, which showed a single peak, was eluted and all samples were pooled.

Enzyme homogeneity. The purified enzyme was dialyzed against distilled water, and the Triton X-100 in the enzyme solution was removed with chloroform.⁸⁾ SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn,⁹⁾ with a 7.5% polyacrylamide gel and phosphate buffer (pH 7.0). Electrophoresis was done at 8 mA per column and gels were stained with Coomassie brilliant blue R-250.

Isoelectric point. Isoelectric-focusing electrophoresis was done by the method of Vesterberg and Svensson¹⁰⁾ using an isoelectrofocusing column (2.5 × 30 cm) (LKB-Produkter, AB, Sweden) and 1% carrier Ampholine (pH 3~10); electrophoresis was done at 4°C and 700 V for 45 hr.

Measurement of molecular weight. The MW of the purified enzyme was measured by gel filtration on a Sephacryl S-300 column (1.5 × 80 cm) by the method of Andrews,¹¹⁾ using a standard protein kit (Pharmacia Fine Chemicals Co., Sweden). SDS-polyacrylamide gel (7.5%) electrophoresis as described above was also used to measure the MW of the enzyme.

Results

Purification of polymyxin acylase from Pseudomonas sp. M-6-3

Because polymyxin acylase is bound to the cell envelope, one of the critical steps in purifying it is solubilizing the enzyme from the cell. As shown in Table I, Triton X-100 treatment gave the best yield of the soluble enzyme, although colistin, a substrate of polymyxin acylase, solubilized the enzyme to a lesser extent. The cell free extract solubilized by Triton X-100 was purified by three-stage chromatography; the results are summarized in Table II. Figure 1A shows the chromatographic profile of the enzyme on a DEAE-cellulose column with a linear gradient of KCl. Using colistin as substrate, 90% of the deacylase activity was detected in the passed solution and 10% in the 500 mM-KCl eluate. The enzyme was further purified twice by hydroxyapatite column chromatography (Fig. 1B) and then twice by Sephacryl S-300 column chromatography. When enzyme homogeneity was assessed by SDS-polyacrylamide gel electrophoresis, a single band of protein was observed (Fig. 2). The properties of the purified

Table I. THE ENZYME ACTIVITY SOLUBILIZED FROM ACETONE-DRIED CELL POWDER BY VARIOUS TREATMENTS

Methods	Treatment time (hr)	Solubilized enzyme activity (units/100 mg powder)
Sonic	0.5	4,000
EDTA-Lysozyme	4	900
Colistin	15	3,600
Triton X-100	15	7,800

Table II. PURIFICATION OF POLYMYXIN ACYLASE FROM *Pseudomonas* sp. M-6-3

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	137.0	47,300	340	100
DEAE-cellulose	64.4	41,900	650	89
1st Hydroxyapatite	23.1	31,200	1,350	66
2nd Hydroxyapatite	19.9	31,000	1,560	66
1st Sephacryl S-300	9.5	16,400	1,720	35
2nd Sephacryl S-300	6.4	11,200	1,750	24

enzyme were as follows:

Stability

The enzyme was stable for only 5 weeks at 4°C, but the presence of 0.1 M ammonium sulfate or 1% bovine serum albumin increase the period of stability to 4 months. Although the enzyme was stable up to 50°C for 24 hr, there was a marked loss of activity after storing at -40°C, and also after incubating at over 60°C for 1 hr.

Optimal pH and temperature

The optimal pH was 9.0 for colistin A and the optimal temperatures of the enzyme was about 45~50°C (although *Pseudomonas* sp. M-6-3 itself cannot tolerate temperatures over 35°C). The observed $V_{\max}$ and K_m values were 1750 nmol/min/mg and 3.85 mM, respectively. For *N*-capryloyl-DL-methionine, these values were 2470 nmol/min/mg and 3.0 mM.

Measurement of MW and isoelectric point

The MW of the purified enzyme was esti-

Fig. 1. Column Chromatography of the Polymyxin Acylase on DEAE-Cellulose (A) and Hydroxyapatite (B).

Eluent: (A) 10 mM phosphate buffer (pH 8.0) and then with a linear gradient from 0 to 500 mM KCl in the buffer; (B) 10 mM phosphate buffer (pH 8.0) and then with a linear gradient from 10 to 500 mM of phosphate buffer; flow rate, 48 ml/hr; fraction, 3 ml/tube; —, absorbance at 280 nm; ●—●, enzyme activity.

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Purified Polymyxin Acylase (Type I).

mated to be 62,000 by gel filtration chromatography on Sephacryl S-300, but occasionally a high molecular weight peak that had the same enzyme activity emerged. The MW calculated by SDS-polyacrylamide gel electrophoresis was 62,000, which agrees well with gel filtration method. The isoelectric point was 5.7, as measured by isoelectric-focusing electrophoresis.

Effects of metal ions, chemical reagents, and inhibitors

The enzyme activity was markedly inhibited by Hg^{2+} and Ag^+ , although the other cations (Mg^{2+} , Ca^{2+} , Mn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , Ba^{2+} , and Pb^{2+} ; 1 mM) had little effect. Metal-ion chelating agents (EDTA, EGTA, *o*-phenanthroline, 8-hydroxy-quinoline) did not inhibit the enzyme over the concentration range 0.1~1.0 mM, but 20 mM EGTA inhibited about 50% of the activity. Neither thiol-protease inhibitors (other than *p*-chloromercuribenzoate) nor serine protease inhibitor (DFP) affected the enzyme activity. TPCK and TLCK did not inhibit. The enzyme

Fig. 3. Effects of Organic Modifiers on the Activity of Polymyxin Acylase (Type I).

The enzyme activity was measured in the presence of various concentrations of organic modifiers by assay method 2. ○—○, methanol; ●—●, ethanol; △—△, ethylene glycol; ▲—▲, *N,N'*-dimethylformamide.

Table III. SUBSTRATE SPECIFICITY OF POLYMYXIN ACYLASE (TYPE I)

Substrate	Relative activity (%)
Acetyl-(C ₂)-DL-methionine	3.0
Butyryl-(C ₄)-DL-methionine	2.3
Caproyl-(C ₆)-DL-methionine	64
Capryloyl-(C ₈)-DL-methionine	100
Capryloyl-(C ₈)-DL-aspartic acid	122
Capryloyl-(C ₈)-DL-glutamic acid	131
Capryloyl-(C ₈)-DL-glycine	116
Capryloyl-(C ₈)-DL-alanine	111
Capryloyl-(C ₈)-DL-valine	105
Capryloyl-(C ₈)-DL-leucine	83
Capryloyl-(C ₈)-DL-serine	98
Capryloyl-(C ₈)-DL-threonine	95
Capryloyl-(C ₈)-DL-phenylalanine	83
Caprinoyl-(C ₁₀)-DL-methionine	107
Lauroyl-(C ₁₂)-DL-methionine	100
Myristoyl-(C ₁₄)-DL-methionine	85
Palmitoyl-(C ₁₆)-DL-methionine	56
Benzoyl-DL-methionine	0.36
Benzoyloxycarbonyl-DL-methionine	0.25

was inhibited by two oxidizing agents, *N*-bromosuccinimide and potassium ferricyanate.

Tolerance for organic modifiers

The enzyme showed tolerance for several

Table IV. DIFFERENCE OF SUBSTRATE SPECIFICITY OF POLYMYXIN ACYLASES (TYPES I AND II) ON SEVERAL *N*-BENZYLOXYCARBONYL AMINO ACIDS

Substrate	Relative activity (%)	
	Type I	Type II
Capryloyl-DL-methionine	100	62
Z-DL-Glutamic acid	5.7	18
Z-DL-Aspartic acid	2.9	8
Z-DL-Methionine	0.25	0.3
Z-DL-Glycine	0.2	0.6
Z-DL-Alanine	0.2	0.6
Z-DL-Tyrosine	0.1	0.2

Table V. REMOVAL OF *N*-BENZYLOXYCARBONYL GROUP USING POLYMYXIN ACYLASE (TYPE II)

The reaction mixture (containing 170 units of enzyme) was incubated at 37°C for 48 hr and the removal of the Z group was confirmed by assay method 3.

Substrate	
Z-Glu(OBu ¹)-Ser-Thr-Leu	+
Z-Ser-Glu(OBu ¹)-Lys(Boc)-Ser-Glu-Thr-Pro	+
Z-Gly-Gly	+
Z-Gly-Gly-NH ₂	+
Z-Gly-Phe	+
Z-Ala-Thr-NH ₂	+
Z-Pro-Ser-Asp(OBu ¹)-Thr-Pro-Ile-Leu-Pro-Gln-OBu ¹	—
Z-Pro-Glu(OBu ¹)-Ile	—

+, completely removed; —, not removed.

organic modifiers such as methanol, ethanol, ethylene glycol, and dimethyl formamide (Fig. 3). Even in 50% ethylene glycol buffer, half of the activity remained.

Substrate specificity

Interestingly, this enzyme had a broader substrate specificity than was expected; for example, it hydrolyzed all of the *N*-acyl D- and L-amino acids that were studied. In the case of *N*-acyl methionines, the enzyme was particularly active for long chain fattyacyl groups, especially capryloyl (C₈), caprinoyl (C₁₀), and lauroyl (C₁₂) groups, but also hydrolyzed *N*-acetyl and *N*-benzoyl DL-methionine slightly (Table III). The activity of the enzyme was equivalent for all *N*-capryloyl amino acids,

regardless of the amino acid residues (Table III).

On the other hand, when the enzyme was solubilized by the Triton X-100 method in 0.2M KCl-containing buffer and was purified in the same way, a single homogeneous protein could not be obtained, and this only partially purified preparation showed a slightly different specificity pattern. The relative activity increased for Z-amino acids (Table IV). We called this enzyme preparation Type II to distinguish it from the enzyme solubilized without KCl (Type I). Despite its rather weak activity, a large amount of the Type II enzyme was able to split the Z group of several Z-peptides much more readily than the Type I could. The results are summarized in Table V.

Discussion

Since Schmiedberg¹²⁾ observed enzymatic hydrolysis of *N*-benzoyl-glycine in the late 19th century, many kinds of enzymes which are able to hydrolyze *N*-acyl amino acids have been reported. In the early stages, interest centered around the optical resolution of racemic amino acids, as exemplified by the work of Greenstein *et al.* with hog kidney acylases,¹³⁾ Kameda *et al.* with bacterial acylases,¹⁴⁾ and Chibata *et al.* with fungus acylases.¹⁵⁾ In the late fifties, interest shifted to penicillin-deacylating enzyme(s) and their use in preparing semisynthetic penicillin. Rolinson *et al.*¹⁶⁾ and Kameda *et al.*¹⁷⁾ published some of the earliest reports; this was followed by a spate of related reports. An earlier paper of ours¹⁾ reported the isolation of a polymyxin-deacylating enzyme (polymyxin acylase) in the cell-bound form and its use in preparing deacyl colistin. The cell-bound enzyme deacylated not only polymyxins but also various *N*-fatty acyl peptides and *N*-acyl amino acids. The substrate specificity of the cell-bound enzyme distinguishes it from other aminoacylase, which usually can hydrolyze *N*-acyl amino acids but not *N*-acyl peptides. In these experiments, we solubilized and purified this new enzyme. We found that the substrate, colistin, is able

to solubilize the cell-bound enzyme; this is consistent with Teuber's observation that polymyxin-lysozyme caused spheroplast formation in *Salmonella*.¹⁸⁾ A suspension of the acetone-dried cell powder of *Pseudomonas* sp. M-6-3 was effective as the enzyme source because of the detergent-activity of colistin on the cell-bound enzyme. The solubilization ability of Triton X-100 was even higher than that of colistin. The solubilizing data in Table I suggest that this enzyme is periplasmic.

After purifying the solubilized enzyme by three-stage column chromatography, we identified it as a single protein by SDS-polyacrylamide gel electrophoresis. The enzyme had a molecular weight of approximately 62,000 and in the presence of Triton X-100, it was liable to polymerize. The properties (substrate specificity, pH-stability, heat tolerance, etc.) were very similar to those of the cell-bound enzyme, although the optimum pH of the purified enzyme was slightly higher. Like *N*-long chain acyl aminoacylase from *Pseudomonas diminuta*,¹⁹⁾ chelators such as EDTA and 8-hydroxyquinoline showed no effects on enzyme activity. This is consistent with the inertness of metal ions toward enzyme activity. Also serine protease inhibitors were not active. The activity of polymyxin acylase was not affected by various sulfhydryl agents other than *p*-chloromercuribenzoate.

The most interesting property of this enzyme(s) was its broad substrate specificity. Especially, the specificity for the amino moiety is not exact; for example the activities for *N*-capryloyl amino acids were indistinguishable, regardless of the amino acid residues; it acted not only on the L-form but also on the D-form, and L-form was more easily hydrolyzed. Furthermore, even *N*-capryloyl derivatives having an amino moiety, such as glucosamine, were hydrolyzed by this enzyme.²⁰⁾ This polymyxin acylase is particularly active on long chain fattyacyl groups, like that of amino acylase from *Mycobacterium phlei*²¹⁾ or *Pseudomonas dismuta*.¹⁷⁾ Interestingly, different conditions in enzyme solubilization gave different specificity patterns for the solubilized

enzyme. "Type I enzyme," solubilized without KCl, was more active towards *N*-fattyacyl amino acids; while "Type II enzyme," solubilized with KCl, was more active for *Z*-amino acids. The mechanism of the formation of Type II is not clear. At present, there are two possible explanations of these results. The first would postulate the existence of both types of enzymes in the cell-bound form. The second possibility is that Type I and Type II enzymes originate from the same protein in the cell. The solution of this problem requires the purification of both types and the determination of their amino acid sequences.

Recently, Soda *et al.*²²⁾ purified, crystallized, and characterized a kind of amino acylase from *Bacillus stearothermophilus*. Their enzyme contains 2 g atoms of zinc per mole of protein, however polymyxin acylase (Types I and II) contains calcium, and its substrate specificity is far from that of their enzyme. Murao *et al.*²³⁾ reported some papers concerning urethane-hydrolyzing enzymes from *Lactobacillus fermenti*, but their enzymes did not act on *Z*-peptides. Polymyxin acylase (Type II) removed the *Z* group of some *Z*-peptides. We believe that both types of polymyxin acylase will have many practical applications in peptide chemistry; two obvious examples are in applying the Edman degradation to naturally occurring *N*-acyl peptides, and in preparing analogs of *N*-acyl peptides.

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