PURIFICATION OF PENICILLIN AMIDOHYDROLASE, AN ENZYME FOR SEMISYNTHETIC PROCEDURES

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Received November 29, 1991
Accepted February 3, 1992

Penicillin amidohydrolase (EC 3.5.1.11.) is one of the few enzymes used successfully for deprotection of primary amino groups of semisynthetic peptides. The available material is usually contaminated by endo- and exopeptidases. We managed to prepare the enzyme devoid of trypsin- and chymotrypsin-like activities using affinity chromatography with specific ligands: Gly-D-Phe-Phe-Tyr-Thr-Pro-Lys-Thr (the fF peptide) and Leu-Gly-Val-D-Arg-Arg-Gly-Phe (the rR peptide). For further purification of the enzyme affinity chromatography with N-phenylacetyl-D-tert-Leu as a ligand was used.

Penicillin amidohydrolase (penicillin G acylase) (PA) from *E. coli* (EC 3.5.1.11.) was among the first enzymes used in semisynthesis of peptides[1,2]. It cleaves off the phenylacetic acid from penicillin G (refs[3,4]) and several amines[5]. The enzyme is stereoselective towards L-configuration[6]. The use of PA for cleaving off the phenylacetic acid from peptides and proteins is complicated by the presence of trypsin- and chymotrypsin-like activities. Most of the authors, who isolated PA, did not consider possible contamination of PA by proteolytic activities[7 - 9]. We removed these proteolytic activities by affinity chromatography using the specific peptides as ligands.

EXPERIMENTAL

Materials

The crude samples of PA, isolated from *E. coli* cells[10], and penicillin G (benzyl*penicillin) were a generous gift of the Research Institute of Biofactors and Antibiotics (Roztoky). Sephadex G-200 and DEAE-Sephacel were purchased from Pharmacia (Uppsala, Sweden). Affinity chromatography was performed on a porous glass carrier with 3-(2-aminoethyl)propyltriethoxysilanol residues as the active groups (gift of Dr M. Čapka from the Institute of Chemical Process Fundamentals, Prague). The chromogenic substrates,

* The nomenclature and symbols of amino acids and peptides obey the published recommendations[11].

Bz-L-Arg-pNA and Suc-L-Phe-pNA were a generous gift of Dr E. Kasafírek from the Research Institute of Pharmacy and Biochemistry, Prague. Gly-D-Phe-Phe-Tyr-Thr-Pro-Lys-Thr (fF peptide) was prepared by the method of Inouye[12,13] and Leu-Gly-Val-D-Arg-Arg-Gly-Phe (rR peptide) was prepared by the method of Bláha[14,15]. N-Phenylacetyl-D-tert-Leu was prepared in the Institute of Organic Chemistry and Biochemistry, Prague. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was supplied by Calbiochem (San Diego, U.S.A.). All other chemicals were purchased from Lachema (Brno, Czechoslovakia) and were of analytical grade.

Methods

The PA activity was estimated using penicillin G as a substrate[16]. The concentration of 6-aminopenicillanic acid, liberated from penicillin G by PA, was estimated spectrophotometrically after the reaction with *p*-dimethylaminobenzaldehyde in acidic solution. The PA activity was estimated at 20 °C as follows: 0.1 ml of the sample was added to 0.9 ml of penicillin G ($5.4 \cdot 10^{-2}$ mol l^{-1}) in 50 mM sodium phosphate buffer pH 7.6. After 15 min, the solution was mixed with 1 ml of *p*-dimethylaminobenzaldehyde ($3.35 \cdot 10^{-2}$ mol l^{-1}) in methanol and 6 ml of 2.3 M sodium acetate buffer pH 2.5. The yellow color was allowed to develop another 10 min and the absorbance at 415 nm was measured. One activity unit was defined as the enzyme amount able to release one μmol of 6-aminopenicillanic acid from penicillin G in one minute.

The relative trypsin- and chymotrypsin-like activities were determined using chromogenic substrates Bz-L-Arg-pNA and Suc-L-Phe-pNA, respectively. The sample (0.1 ml) was mixed with 0.9 ml of 50 mM sodium phosphate buffer pH 7.6 and with 1 ml of Suc-L-Phe-pNA or Bz-L-Arg-pNA ($5 \cdot 10^{-4}$mol l^{-1}) in 5% N,N-dimethylformamide. The reaction was allowed to proceed for 48 h at 37 °C in the dark. The absorbance of the resulting solution at 405 nm was taken as a measure of the proteolytic activity.

The chromatography on DEAE-Sephacel and gel chromatography on Sephadex G-200 were employed as the first steps to purify the crude samples of PA (marked A). The chromatography was performed on DEAE-Sephacel column (3.8 x 7 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.6. The flow rate was 1 ml min^{-1}. Bound proteins were eluted with 0.5 M NaCl in the elution buffer. PA active fractions were further purified on a Sephadex G-200 column (4.5 × 56 cm) in the same buffer. The flow rate was 0.3 ml min^{-1}. PA active fractions (marked A_1) were pooled and freeze dried. Both chromatographies were performed at 4 °C.

fF and rR peptides (20 mg of each) were coupled to the carrier (1.5 g) with the use of glutaraldehyde[17]. The PA was purified on columns (1 × 4 cm) with these carriers equilibrated with 50 mM sodium phosphate buffer pH 7.6. The sample (4 mg of A_1) in 1 ml of the elution buffer was applied to each column at a flow rate of 1 ml min^{-1} under laboratory temperature. Bound proteins were eluted by 0.5 M NaCl in the same buffer. The fractions of 1 ml were collected and analyzed for tryptic and chymotryptic activity. The isolated PA was marked A_2 and A_3 (from the carrier with fF and rR ligand, respectively).

The N-phenylacetyl-D-tert-Leu ligand (95 mg) was coupled to the carrier (1 g) using water-soluble carbodiimide[18]. N-Phenylacetyl-D-tert-Leu (100 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (177 mg) in 10 ml of 75% ethanol (acidified to pH 5.1 with HCl) were stirred for 24 h at room temperature. The carrier with the ligand was well washed with acidified ethanol, water, 1 M NaCl in 50 mM sodium phosphate buffer pH 7.6 and with water. Affinity chromatography was performed in a glass column (1 × 4 cm) in 50 mM sodium phosphate buffer pH 7.6. The sample (10 mg of A_2) was applied to the column in a volume of 1 ml at a flow rate of 1 ml min^{-1} under laboratory temperature. Bound proteins were eluted by 25 mM phenylacetic acid in the elution buffer. The fractions of 1 ml were collected and checked for PA activity. The isolated PA was marked A_4. The concentration of proteins was determined according to Bradford[19].

RESULTS AND DISCUSSION

The crude sample of PA (A) was prepurified by ion exchange chromatography on DEAE-Sephacel and by chromatography on Sephadex G-200. Most of the contaminating proteins can be efficiently removed by combination of these methods. The obtained PA preparation (A_1) was subjected to affinity chromatography in order to remove trypsin- and chymotrypsin-like activities (Fig. 1 and Fig. 2). Both activities are significantly retarded by the fF ligand (Fig. 1). The rR ligand exhibits higher affinity to tryptic activity, the chymotryptic one is retarded to a lesser extent (Fig. 2). The fF peptide proved to be more efficient in this respect. The heterogenous composition of both contaminating proteolytic activities is also apparent from the elution profiles. Significant difference was not found between preparations A_2 and A_3 (isolated with the use of fF and rR ligand, respectively) (Fig. 1 and Fig. 2). The preparation A_2 cleared of the most of trypsin- and chymotrypsin-like activities was then purified by affinity chromatography with N-phenylacetyl-D-tert-Leu ligand. This step was very efficient in the respect to the PA activity (preparation A_4) (Fig. 3). A part of the PA activity was not

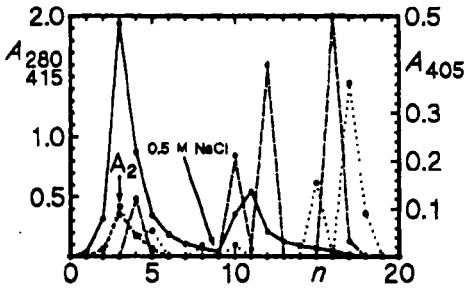

FIG. 1
Affinity chromatography of PA (A_1) on the carrier with fF peptide: PA activity (A_{415} - - - -), tryptic activity (A_{405} — — — —), chymotryptic activity (A_{405} ·····), proteins (A_{280} ——), fraction number n. The isolated PA (A_2) fraction is marked by the arrow

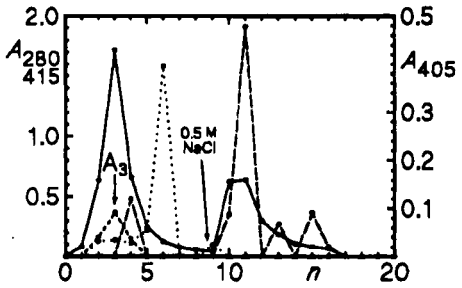

FIG. 2
Affinity chromatography of PA (A_1) on the carrier with rR peptide: PA and proteolytic activities are marked as in Fig. 1. The isolated PA (A_3) fraction is marked by the arrow

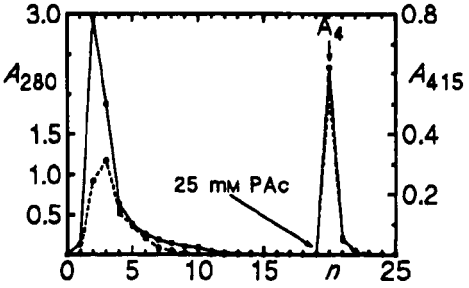

FIG. 3
Affinity chromatography of the sample A_2 on the carrier with N-phenylacetyl-D-tert-Leu ligand. Activity of PA (A_{415} - - - -), protein concentrations (A_{280} ——), fraction number n. The isolated PA (A_4) is marked by the arrow

retained on the carrier, as a result of low column capacity. If smaller samples were applied the peak of non-retained PA activity decreased to a corresponding extent but the peak of retained PA activity remained the same. The results of all purifications steps are summarized in Table I.

TABLE I
Purification of PA from *E. coli*

PA [a] preparation	Specific PA activity, U / mg	Relative recovery
A	15	100.00
A_1	72	26.00
A_2	124	0.70
A_3	122	0.65
A_4	214	0.35

[a] A crude PA; A_1 preparation isolated by gel chromatography on Sephadex G-200; A_2 and A_3 preparations isolated by affinity chromatography with fF and rR ligand, respectively; A_4 preparation isolated with the use of N-phenylacetyl-D-tert-Leu ligand.

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Translated by P. Hrbas.