Purification and Properties of Pantothenase from *Pseudomonas fluorescens*

By R. KALERVO AIRAS, EERO A. HIETANEN and VEIKKO T. NURMIKKO
Department of Biochemistry, University of Turku, 20500 Turku 50, Finland

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Pantothenase (EC 3.5.1.22) from *Pseudomonas fluorescens* UK-1 was purified to homogeneity as judged by disc-gel electrophoresis and isoelectric focusing. The purification procedure consisted of four steps: DEAE-Sephadex chromatography, $(\text{NH}_4)_2\text{SO}_4$ precipitation, hydroxyapatite chromatography and preparative polyacrylamide-gel electrophoresis. Gel filtration on Ultrogel AcA 34 was used to determine the molecular weight, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to study the subunit molecular weight. The enzyme appeared to be composed of two subunits with mol.wts. of approx. 50000 each. The total mol.wt. of the enzyme was thus about 100000. The isoelectric point was 4.7 at 10°C.

Pantothenase (pantothenate amidohydrolase, EC 3.5.1.22) catalyses the degradation of pantothenate into pantoate and β -alanine. In the classification of aerobic pseudomonads proposed by Stanier *et al.* (1966), only the strains of *Pseudomonas fluorescens* biotype C were capable of growing with pantothenate as the sole source of carbon and nitrogen. Pantothenase is the first enzyme in the degradation of pantothenate (Goodhue & Snell, 1966). It was partially purified from *Ps. fluorescens* P-2 in this laboratory by Nurmikko *et al.* (1966), and highly purified by Puisto & Nurmikko (1970). Successful use of a similar purification procedure proved, however, impossible in purifying the enzyme from *Ps. fluorescens* UK-1; in the present paper an appropriate method for this procedure is presented.

Pantothenase from *Ps. fluorescens* UK-1 has a special property of almost total reactivation after thermal inactivation in whole cells (Airas, 1972). The study of this reactivation was really the immediate reason for the development of the new purification method. The purified enzyme gave one band in polyacrylamide disc-gel electrophoresis and in sodium dodecyl sulphate/polyacrylamide disc-gel electrophoresis. Also some properties of the enzyme are reported, including its molecular weight and subunit structure.

Materials and Methods

Bacterial strain and media

The cultivation of *Ps. fluorescens* UK-1 was performed as described by Airas (1972) with the following exceptions. The medium used was void of $(\text{NH}_4)_2\text{SO}_4$, 10 mM-potassium pantothenate was used to supply the necessary carbon and nitrogen and 10 mM-glucose to supply an additional carbon

source. The final cultivation was carried out in a 150-litre fermentor. The cells were collected when their fresh weight reached 1.5 mg/ml and stored at -20°C .

Reagents

Deoxyribonuclease was from Miles-Seravac (Pty) Ltd., Maidenhead, Berks., U.K.; bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, MO 63178, U.S.A.; hydroxyapatite was Bio-Gel HT purchased from Bio-Rad Laboratories, Richmond, CA 94804, U.S.A.; Ultrogel AcA 34 was obtained from LKB, Bromma, Sweden.

Enzyme assay

Pantothenase activity was determined as described previously (Airas, 1972), with a few exceptions. The reaction mixture (125 μl by total vol.) contained 30 mM-potassium $[1-^{14}\text{C}]$ pantothenate (80000 d.p.m./125 μl), 20 mM-potassium phosphate, pH 7.2, and a sample of pantothenase. The reaction temperature was 20°C . The reaction was stopped by pipetting samples (25 μl) of the reaction mixture on to Whatman no. 1 chromatography paper, located 1 cm from a surface of 30% (v/v) formic acid. The chromatograms were developed in butan-2-one/methanol/water/36% HCl (60:15:15:1, by vol.). (R_F values: β -alanine, 0.30; pantothenate, 0.85).

Protein determination

Koch & Putnam's (1971) modification of the biuret method was used to determine the protein concentrations with bovine serum albumin as standard protein.

Molecular-weight determination by gel filtration

A column (1 cm $\times$ 40 cm) of Ultrogel AcA 34 was equilibrated with the elution buffer, 50 mM-potassium phosphate, pH 7.5. The sample contained, in 0.5 ml,

the pantothenase and the standard enzymes (mol.wt.): aldolase (158000), alcohol dehydrogenase (150000), lactate dehydrogenase (140000), glucose 6-phosphate dehydrogenase (128000), alkaline phosphatase (86000), malate dehydrogenase (70000) and haemoglobin (64000). Fractions (nine drops; 0.3 ml) were collected. The standard enzymes were assayed by standard techniques as described by Boehringer Mannheim G.m.b.H. (1972); haemoglobin was measured at E_{405} . The standard enzymes gave a straight line when elution volume was plotted against log (molecular weight).

Polyacrylamide disc-gel electrophoresis

The method of Davis (1964) was performed on a 7.5% (w/v) polyacrylamide gel lacking the spacer and sample gels, but with 10% (w/v) sucrose in the sample solution. The gels were then stained with Coomassie Brilliant Blue R as advocated by Weber *et al.* (1972).

Preparative polyacrylamide disc-gel electrophoresis was carried out in an apparatus manufactured by Stålprodukter, Uppsala, Sweden. It was essentially performed as for analytical disc-gel electrophoresis. The gel size was 120 mm × 10 mm, and the run was carried out at 4°C. The elution was done with the electrode buffer, as usual with the above equipment.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This electrophoresis was carried out by the method of Weber *et al.* (1972). The standards (subunit mol. wts. in parentheses) were lysozyme (14000), glyceraldehyde 3-phosphate dehydrogenase (36000), aldolase (40000), glutamate dehydrogenase (53000), catalase (58000) and bovine serum albumin (68000). The standards gave a straight line when plotted as log (molecular weight) versus mobility.

Isoelectric focusing

Analytical thin-layer electrofocusing in polyacrylamide gel was performed in a LKB Multiphor apparatus. Commercial thin-layer gels, Ampholine PAG plate (LKB, Bromma, Sweden) pH 3.5–9.5 were used, and the manufacturer's directions were followed in the run and staining of the gel.

Results

Purification of pantothenase

(a) *Disruption of cells.* A cellular mass (108 g) of *Ps. fluorescens* UK-1 was suspended in 210 ml of buffer 1 (see under DEAE-Sephadex chromatography); the obtained suspension was then compressed through a French press.

(b) *Deoxyribonuclease treatment.* $MgCl_2$ (5 mM) and 10 μ g of deoxyribonuclease/ml were added to the cell extract, which was then incubated at 20°C for 30 min; during this period a rapid fall of viscosity was

observed. The enzyme solution was then centrifuged at 10000g for 20 min. The decomposition of DNA was necessary to ensure the successful performance of the ion-exchange chromatography.

(c) *DEAE-Sephadex chromatography.* Pantothenase was found to attach effectively to DEAE-Sephadex even below pH 6, suggesting a comparatively acid protein. This observation was utilized during the purification process; most cellular proteins could be eluted through the ion-exchanger with the pantothenase remaining attached.

Buffers: buffer 1 contained 20 mM-piperazine, 30 mM-KCl and 10 mM-potassium pantothenate. pH was adjusted to 5.9 (at 20°C) with 5M-HCl. Pantothenate served as a protective agent, and KCl was added to increase Cl^- ion concentration. Piperazine ($pK = 5.3$) might be substituted for histidine ($pK = 6.0$) or 2-picoline ($pK = 6.0$). Buffer 2 was obtained by adding 30 mM- $(NH_4)_2SO_4$ to buffer 1; buffer 3 by adding 500 mM- $(NH_4)_2SO_4$ to buffer 1.

The column containing 6 g of DEAE-Sephadex A-50 was equilibrated with buffer 1. The centrifuged enzyme solution was applied to the column, and the first big, turbid peak containing most of the cellular material was eluted with 700 ml of buffer 2. Pantothenase was eluted from the column by increasing the $(NH_4)_2SO_4$ concentration. The gradient was logarithmic; buffer 3 was led to the mixing chamber con-

Fig. 1. DEAE-Sephadex column chromatography of pantothenase

The column (4 cm × 18 cm) was equilibrated with buffer 1 (piperazine/HCl, pH 5.9, see the text). The extract (about 200 ml) was applied to the column and elution was begun with 700 ml of buffer [containing 30 mM- $(NH_4)_2SO_4$]. Thereafter a gradient of $(NH_4)_2SO_4$ was started. Buffer 3, containing 500 mM- $(NH_4)_2SO_4$, was led into a constant-volume mixing chamber with 500 ml of buffer 2. Fractions (15 ml) were collected and E_{280} (●) and pantothenase activity (○) were measured from diluted samples.

taining 500ml of buffer 2. The duration of the whole process was 10h and it was carried out at 8°C. E_{280} was measured on diluted fraction samples. In Fig. 1 the elution profile is shown.

(d) $(\text{NH}_4)_2\text{SO}_4$ precipitation. Pantothenase is precipitated between 1.35–1.55M- $(\text{NH}_4)_2\text{SO}_4$ at 0°C (about 35–40% satn.). The enzyme peak from DEAE-Sephadex chromatography was collected; 20mM-Tris was added to raise pH, and the enzyme was precipitated by adding 1.8M- $(\text{NH}_4)_2\text{SO}_4$. The final $(\text{NH}_4)_2\text{SO}_4$ concentration was about 2.0M (50% satn.). The precipitate was left for 18h at 0°C then centrifuged for 15min at 7000g and dissolved in 6ml of 35mM-potassium phosphate, pH6.8. Excess of $(\text{NH}_4)_2\text{SO}_4$ was then removed by gel filtration through a column (2cm × 30cm) of Sephadex G-25 (coarse grade). The run was done upwards; the flow was achieved by using a peristaltic pump, and 35mM-potassium phosphate, pH6.8, served as buffer.

(e) Hydroxyapatite chromatography. The desalted enzyme solution was applied to the column (3cm × 3cm) of hydroxyapatite, and the elution was carried out with 35mM-potassium phosphate, pH6.8. The eluted enzyme (Fig. 2) was collected and precipitated by adding 2.5M- $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged at 7000g for 15min, dissolved in 2–3ml of the electrode buffer used in disc-gel electrophoresis, and dialysed against the electrode buffer. The adequate phosphate concentration in hydroxyapatite chromatography was found by a previous run with a linear gradient from 10mM-potassium phosphate.

(f) Preparative polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was applied as the last stage in the purification process. The equipment supplied by Stålprodukt was used as described in the Materials and Methods section. The gel dimen-

Fig. 3. Preparative disc-gel electrophoresis of pantothenase

The apparatus of Stålprodukt was used, and the method was essentially the standard system of Ornstein & Davis (Davis, 1964). Gel size was 10mm × 120mm, the sample (3ml, 20mg of protein) contained 10% sucrose. The run was for 12h, and the current was maintained at 12mA. The elution was begun just before the tracking dye (Bromophenol Blue) came out of the gel, the elution flow rate was 20ml/h and fractions (2ml) were collected. An Isco model UA-2 was used to monitor E_{280} (---) and the enzyme activities (○—○) were measured. The first, big absorption peak was the dye. Staining of the gel after the run still showed three protein bands in the gel.

sions were 1cm × 12cm, and no more than 20mg of protein was loaded on to the column. The elution profile is presented in Fig. 3.

Table 1 summarizes the purification process of pantothenase. The final specific activity was 220 nkat/mg, and the yield after preparative disc-gel electrophoresis was about 17%.

Criteria of purity

The purity of the pantothenase preparation was established by analytical disc-gel electrophoresis, by isoelectric focusing in polyacrylamide gel, and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Only one band is derived by all three methods from the purified pantothenase preparation. Some tailing is observed in electrofocusing gels below pH5 (without sharp bands), and this may be caused by the instability of pantothenase at this pH. The enzyme purified with hydroxyapatite contains less than 10% of other protein as estimated from the intensities of the disc-gel bands. The disc-gel band of the enzyme inactivated at 40°C was identical with that of native enzyme. This may indicate that the final purified enzyme contains an unknown amount of inactivated forms of the enzyme. The $(\text{NH}_4)_2\text{SO}_4$ precipitation of a highly purified enzyme regularly resulted in a considerable increase of specific activity, with no apparent indications of increased purification being observed with disc-gel electrophoresis. This suggests that the inactivated form of the enzyme can be partly separated from the native form by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The specific activity of hydroxyapatite-purified enzyme was increased to 270 nkat/mg by $(\text{NH}_4)_2\text{SO}_4$ precipitation, but the band of impure material still remained.

Fig. 2. Hydroxyapatite chromatography of pantothenase

The column (3cm × 3cm) was equilibrated with 35mM-potassium phosphate, pH6.8. The enzyme solution (21ml) was applied to the column and eluted with the above buffer. Protein in the effluent was followed with an Isco model UA-2 monitor, and the elution was stopped after the first peak of material had eluted from the column. Fractions (5ml) were collected and E_{280} (●) and pantothenase activity (○) were measured from diluted samples.

Table 1. Purification of pantothenase from *Ps. fluorescens*

Fraction	Volume (ml)	Total protein (mg)	Total activity (μ kat)	Specific activity (nkat/mg)	Recovery (%)
Cell extract	311	7060	29.4	4.17	(100)
After deoxyribonuclease treatment and centrifugation	301	4570	26.3	5.77	90
Pooled fractions from DEAE-Sephadex	280	954	18.1	19.0	62
After $(\text{NH}_4)_2\text{SO}_4$ precipitation	21.2	194	13.4	69.5	46
Pooled fractions from hydroxyapatite	82	67	8.25	124	28
Sample to preparative disc gel	3.0	6.4	0.80	124	(100)
Preparative disc-gel eluate	11.6	2.2	0.48	220	60

Molecular weight and subunit structure

The molecular weight of pantothenase was determined by gel filtration on a column of Ultrogel AcA 34 by using commercial enzymes as standards. The mean value of the mol.wt. received from six runs was $97\,400 \pm 4800$ (S.D.).

The molecular weight of the subunits of pantothenase was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The mean result of four determinations was $50\,000 \pm 700$ (S.D.). On the basis of these determinations it seems obvious that pantothenase is composed of two subunits of equal size, and that the total mol.wt. is approx. 100000.

Isoelectric point

The isoelectric point of pantothenase was determined by analytical thin-layer gel electrofocusing in polyacrylamide gel. The isoelectric point was 4.7 at 10°C.

Stability

Pantothenase is rather susceptible to heat both in whole cells and in purified form. Heat inactivation, which will later be given a more detailed consideration, occurs at temperatures above 28°C. The purified enzyme loses part of its activity when frozen. In 5mM- K_2SO_4 the loss of activity is 45%; in 50mM-potassium phosphate, pH7.0, it is 20% for one round of freezing and thawing. Pantothenate (substrate) and oxalate (inhibitor) protect it from inactivation. Freezing is, however, an excellent way of storing the enzyme. Pantothenase is stable between pH5.5–10.0, but with pH values below 5 its activity is rapidly decreased.

U.v.-absorption spectrum

The u.v.-absorption spectrum of purified pantothenase resembles that of tryptophan (Fig. 4). An absorption maximum was found at 282nm and shoulders at 276 and 291 nm. The specific extinction coefficient, $E_{1\text{cm}}^{1\%}$ = 16, but that of pure active panto-

Fig. 4. U.v.-absorption spectra of pantothenase

The spectra were recorded by Unicam SP.800 spectrophotometer with slit-width of 0.5mm. Pantothenase concentration was 0.4 mg/ml. Curve 1, pantothenase in 50mM-potassium phosphate, pH7.0. Curve 2, the pH of the solution in curve 1 was adjusted to 12.0 with 5M-KOH. Curve 3, spectrum of L-tryptophan with same settings of the spectrophotometer.

thenase may be slightly different because absorption depends on the degree of denaturation. By using the method developed by Bencze & Schmid (1957), the tyrosine/tryptophan molar ratio can be determined from the slope of the tangent of the absorption curve recorded at pH12, and is 1.08. Further, Edelhoch (1967) has described a method of determining the amount of tryptophan residues from the spectrum measured in the presence of 6M-guanidine hydrochloride. The amount of the pantothenase tryptophan residues, thus determined, was 26, the amount of tyrosine residues consequently 28.

Discussion

The purification method described above has been successfully re-used in the preparation of pure panto-

thenase. The basic stage of purification, the DEAE-Sephadex chromatography, has proved very reproducible; the low isoelectric point of the enzyme facilitates its separation by an ion-exchanger from most other proteins. Preparative disc-gel electrophoresis, which forms the last stage of purification, is the weakest point of this purification procedure. Pure enzyme is obtained, but the amount of the enzyme that can be treated at one single run is small.

The isolation of the pantothenase from the UK-1 strain has so far proved impossible by using the isolation method that Puisto & Nurmikko (1970) used on the P-2 strain, although strains P-2 and UK-1 actually represent the same species of *Ps. fluorescens*. This method consisted of four stages: precipitation at pH 5, $(\text{NH}_4)_2\text{SO}_4$ precipitation, hydroxyapatite chromatography and preparative disc-gel electrophoresis. Precipitation at pH 5 cannot be used on the pantothenase from strain UK-1 since it is rapidly inactivated at this pH. The pantothenase of strain P-2 was precipitated with 40–60% satd. $(\text{NH}_4)_2\text{SO}_4$, but 40% satn. precipitated most of the strain UK-1 pantothenase. The attachment of the pantothenase from strain P-2 to hydroxyapatite is considerably weaker than that of the enzyme from strain UK-1, because the former was eluted from the gel with 5 mM-phosphate, and the latter with 35 mM-phosphate. These differences in enzyme precipitation, adsorption and stability are bound to imply differences in the enzyme structures as well. The enzyme kinetics also reveal certain differences; in strain P-2 the K_m value is lower than in the UK-1 strain (Nurmikko *et al.*, 1966; Airas, 1976).

The final specific activity obtained (220 nkat/mg) seems to be rather low but is, however, of the same order of magnitude as that of several other purified

amidases, e.g. commercial proteases. The specific activity was lower than the value (1450 nkat/mg) obtained for the enzyme of strain P-2 (Puisto & Nurmikko, 1970). The gap between the two values is intensified, to a certain degree, by the differences in determination temperatures and determination pH, but even considering these, the specific activity of the pantothenase from strain UK-1 remains only one-third of that from strain P-2. The amount of inactivated pantothenase in the final enzyme preparation is unknown and rather difficult to clarify. Although the specific activity is increased by $(\text{NH}_4)_2\text{SO}_4$ precipitation, it remains uncertain how completely the inactivated form is separated.

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