Isolation of an Inducible Amidase from *Pseudomonas acidovorans* AE1

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SUMMARY

A bacterial strain, AE1, which hydrolysed acetanilide, was isolated from soil and identified as *Pseudomonas acidovorans*. Numerous amides, esters and enzyme inhibitors were tested as amidase inducers. Phenacetin was chosen as inducer for the large scale cultivation of these organisms because it was less toxic to the bacteria than acetanilide. The induction increased the enzymic activity 250-fold. In comparison, the type culture strain of *P. acidovorans*, ATCC 15668, had no amidase activity which could be induced by phenacetin. Optimal growth conditions were established with respect to the concentration of carbon source and inducer so that about 10 % of the extractable bacterial protein consisted of the amidase. The organisms were lysed with lysozyme in the presence of EDTA and the enzyme was isolated mainly by column chromatography procedures. A preparation from 60 g (wet wt) bacteria yielded about 100 mg highly purified amidase with a specific activity of 137 μmol substrate hydrolysed/min/mg protein. In addition to acetanilide, the purified enzyme hydrolysed several other amides and esters. As standard substrate, *p*-nitroacetanilide was chosen.

INTRODUCTION

During a search for acetanilide-hydroxylating mono-oxygenases in micro-organisms, a bacterial strain was isolated from soil which catalysed the hydrolysis of acetanilide (F. Bernhardt, personal communication). An enzyme with the same activity had previously been isolated from pig liver microsomes (Krisch, 1963*a*), and this proved to be identical to the well-known unspecific carboxylesterase (EC. 3.1.1.1) (Krisch, 1963*b*).

As part of a larger comparative project on the molecular and catalytic properties of esterases, it seemed worthwhile to include studies of a bacterial amidase/esterase.

We report the identification of the acetanilide-hydrolysing strain, its optimal growth conditions and large-scale cultivation. Results of induction studies and the isolation of the amidase to a homogeneous state are also described.

METHODS

Isolation of bacteria. The culture was obtained from F. Bernhardt (Giessen, Germany), who had previously isolated the strain from soil using a mineral salts medium (Ribbons & Evans, 1962) which contained 0.4 % (w/v) acetanilide as sole carbon and energy source.

Growth of cultures. Cultures were maintained on agar slants prepared from Standard

I-Nähragar (Merck, Darmstadt, Germany). Incubation was at 30 °C; fully grown cultures were stored at 4 °C. One litre of culture medium contained: 1 M-phosphate buffer pH 6.8 (40 ml); 'Hutner's vitamin-free mineral base' (Cohen-Bazire, Sistrom & Stanier, 1957) (20 ml); and 10 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution (10 ml). This comprised the 'standard mineral base' as described by Stanier, Palleroni & Doudoroff (1966). Carbon source, inducer and yeast extract (0.1 g/l), were added to this solution except in the tests for utilization of single organic carbon and energy sources. The pH was adjusted to 6.8 before autoclaving, and was found to be between 7.0 and 7.2 afterwards.

Measurement of bacterial growth. As an indicator of growth yield, the extinction at 660 nm (1 cm light path) was measured with a PM4 spectrophotometer (Carl Zeiss, Oberkochen, Germany). Viable counts were obtained from plate counts, and the total number of micro-organisms was determined with a Thoma cell counting chamber. A total number of 10^8 bacteria/ml gave an $E_{1\text{ cm}}$ of 0.094. The relation was linear up to $E_{1\text{ cm}} = 0.8$.

Identification of species. For genus determination *Bergey's Manual of Determinative Bacteriology* (1957) was used. Species identification was carried out according to Stanier *et al.* (1966).

Chemicals. D-Fucose, D-tryptophan and valine were purchased from Serva, Heidelberg, Germany, sodium maleate, geraniol and δ -aminovalerate from Fluka, Eschborn, Germany, and *p*-hydroxybenzoate from Riedel de Haen, Seelze-Hannover, Germany; all other chemicals were from Merck.

Induction experiments. All substances to be tested as possible inducers were added at concentrations of 0.01 to 0.18 % (w/v) to a culture medium containing 0.55 % (w/v) lactic acid. The pH was adjusted to 6.8 with 6 M-NaOH. The bacteria were grown for 72 h at 30 °C and then harvested by centrifuging (30 min, 12000 g). Sediments were stored frozen before testing. The bacterial sediment was disintegrated by lysozyme (see *Disruption of bacteria*, below) and the supernatant liquid was kept refrigerated until the assays of activity and protein could be carried out.

The following substances were tested as inducers. Amides: acetanilide, acetamide, *p*-ethoxyacetanilide (phenacetin), sulphanilamide, nicotinamide, xylocaine, L-leucineamide, L-leucine- β -naphthylamide, L-tyrosyl-tyrosine, glycyl-L-proline, L-leucyl-glycine, glycyl-tyrosine, leucyl-tyrosine, phenylalanyl-phenylalanine, benzoyl-L-arginineamide, monoethyl-glycine-xylylide. Esters: *N*- α -benzoyl-L-arginine-ethylester, *N*-benzoyl-L-tyrosine-ethylester, acetyl-L-tyrosine-ethylester, L-tyrosine-ethylester, L-phenylalanine-methylester, diethyl-aminoethyl-diphenylpropyl-acetate (SKF 525-A). Other substances: aniline, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, naphthalene, β -naphthylamine, xylydine, *p*-ethoxyaniline (phenetidine), 2,6-dimethylaniline.

The phenacetin used for the induction was a product of Merck (No. 7221), and fulfilled the purity requirements of the British Pharmacopoeia 1968 and the Deutsche Arzneimittelbuch DAB 7. The melting point was 135.5 to 136 °C (137 to 138 °C, *Handbook of Chemistry and Physics*, 1969–1970; 134.7 °C, D'Ans-Lax, 1964). A thin-layer chromatogram according to Bernhammer & Krisch (1965) showed only one spot.

Growth of large bacterial quantities. The inoculum was pre-grown for 24 h in a mineral salts medium with 0.22 % (w/v) lactic acid without phenacetin. The pH was adjusted to 6.8 with 6 M-NaOH. The main batch culture was carried out in 5 l flasks containing 4 l of the same medium with 1 g phenacetin/l. These flasks were inoculated with 200 ml of the culture grown without phenacetin. After 72 h of vigorous aeration at 30 °C, the bacteria were harvested by continuous flow centrifuging (Sorvall KSB, I; Sorvall Inc., Norwalk, Connecticut, U.S.A.). The total yield was frozen at -18 °C.

Disruption of bacteria. In preliminary experiments ultrasonic treatment with an 80 W sonifier Type B 12 (Branson Instruments, Inc., Stamford, Connecticut, U.S.A.) was used. The sonified solution was carefully cooled with ice water.

To obtain optimal conditions for lysis the EDTA concentration was varied. In such preliminary experiments the suspensions were incubated for 2 h at 30 °C. After centrifuging, the sediments were not washed. For standard enzyme preparations the bacteria were suspended in 4 ml 0.05 M-phosphate buffer pH 7.5 (containing 0.025 M-EDTA) per gram bacterial wet weight. Lysozyme (15000 u/mg, Merck) was added to a concentration of 0.5 mg/ml. This mixture was then stirred for 16 h at 4 °C and an extract obtained by centrifuging for 30 min at 100000 g. The sediment was washed once with the same volume of distilled water. The pH was kept constant at 7.5. Distilled water for washing, instead of buffer, was necessary for the subsequent removal of nucleic acids. The supernatant liquid was combined with the extract and the resulting solution used for further enzyme purification.

Determination of amidase activity. The hydrolysis of *p*-nitroacetanilide was measured at 405 nm (1 cm light path) in an Eppendorf photometer connected with an Eppendorf recorder (Netheler & Hinz, Hamburg, Germany). The cuvette contained 1 ml substrate solution (10^{-3} M-*p*-nitroacetanilide dissolved in warm water and then cooled to room temperature) and 1 ml 0.1 M-tris buffer pH 8.6 containing the enzyme. The measurements were performed at 30 °C. The molar extinction coefficient of *p*-nitroaniline in 0.05 M-tris buffer pH 8.6 was 10000 l/mol/cm.

Protein determination. The protein concentration was determined using a micro version of the biuret method (Beisenherz *et al.* 1953). Water and protein solution were combined in Eppendorf micro test tubes (Netheler & Hinz) to a total volume of 1 ml. Then 0.2 ml of a 3 M-trichloroacetic acid solution was added. This mixture was shaken for 5 min with an Eppendorf rotation shaker. The denatured protein was then sedimented by centrifuging for 1 min in an Eppendorf microcentrifuge and the supernatant liquid discarded. The sediment was subsequently dissolved in 0.5 ml biuret reagent and 0.5 ml water and measured after 30 min in microcuvettes (2 cm path length) at 546 nm, with bovine serum albumin as standard. After column chromatography, the fractions were measured at 280 nm (0.5 cm light path) for protein determination.

Enzyme isolation. Step 1: Removal of nucleic acids. Streptomycin sulphate was added to the crude extract to a final concentration of 1 g/100 ml. The sample was then centrifuged for 30 min at 12000 g and the precipitate discarded.

Step 2: Fractional precipitation by ammonium sulphate. To the supernatant liquid from step 1, solid ammonium sulphate was slowly added until the solution was 30 % saturated. The sulphate concentration was checked by titration with BaCl₂ (Bergmeyer *et al.* 1961). The reaction mixture was centrifuged for 30 min at 12000 g, the sediment discarded, and further ammonium sulphate added to the supernatant liquid until 65 % saturation was reached. During the precipitation the pH was kept constant at 7.5. After centrifuging for 30 min at 12000 g the sediment was dissolved in 0.005 M-tris buffer pH 8.6 containing 0.001 M-EDTA and 2 % (v/v) glycerol. This buffer will be designated as 'tris buffer for column chromatography'.

Step 3: Column chromatography with DEAE-Sephadex A-50. The enzyme solution was either dialysed or desalted by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) and applied to a DEAE-Sephadex A-50 column (Pharmacia; 5 × 50 cm). The DEAE-Sephadex was equilibrated with tris buffer for column chromatography. A gradient was applied from a 2-vessel gradient system. The first vessel contained 1100 ml tris buffer for column chromatography, and the second vessel contained the same buffer with 0.35 M-

NaCl. The fractions containing amidase activity were combined and dialysed against tris buffer for column chromatography containing ammonium sulphate, the concentration of which had been calculated to yield a final concentration of 65 % saturation after dialysis.

Step 4: Gel filtration. The enzyme precipitate was centrifuged and dissolved in as little phosphate buffer (0.005 M, pH 8.0) as possible. This solution was applied to a column (2.5 × 100 cm) filled with Sephadex G-100 (Pharmacia) pretreated as specified by the manufacturer.

Step 5: Hydroxyapatite. Bio-Gel HTP (Bio-RAD Laboratories, Richmond, California, U.S.A.) was equilibrated with 0.005 M-phosphate buffer pH 8.0. The pooled enzyme (after gel filtration) was applied to a hydroxyapatite column (2.5 × 13 cm). The adsorbed protein was eluted with a linear phosphate buffer gradient pH 8.0 from 0.005 to 0.06 M. Temperature and rate of elution were kept constant (Bernardi, 1971). If necessary the rate of elution was restricted to 1 ml/3 min.

Purity of the enzyme. Analytical disc electrophoresis. This was performed according to Hedrick & Smith (1968) with 4, 6 or 8 % (w/v) polyacrylamide gels. The protein was stained with 1 % amidoblack in 7 % acetic acid and the gels were electrophoretically destained with 7 % acetic acid. Staining of esterase activity with α -naphthyl acetate was performed as described by Heymann, Junge & Krisch (1972).

Reaction with diethyl *p*-nitrophenylphosphate. A 10^{-2} M stock solution of diethyl-*p*-nitrophenylphosphate (purity > 98 %; Bayer, Leverkusen, Germany) was prepared according to Krisch (1966). The absorbance difference was measured in microcuvettes (light path 2 cm) at 405 nm with an Eppendorf photometer. The test tube contained 0.25 ml 0.2 M-tris buffer pH 8.0, 0.05 ml diethyl-*p*-nitrophenylphosphate stock solution and 0.2 ml of a highly purified enzyme solution (containing 0.5 to 1 mg protein/ml). The extinction of buffer, diethyl-*p*-nitrophenylphosphate and enzyme was subtracted from the 'initial burst'. The amount of *p*-nitrophenol was calculated using the extinction coefficient as determined by Krisch (1966): $\epsilon = 16240$ l/mol/cm at pH 8.0.

RESULTS

Species identification

A comparison of the isolate with *Pseudomonas acidovorans* ATCC15668 (kindly provided by Professor R. Y. Stanier) showed the same 'general characters of diagnostic value for the differentiation of species of aerobic pseudomonads' (Stanier *et al.* 1966). Our isolate and the reference strain differed in using phenacetin as the only source of carbon and energy. Strain ATCC15668 did not grow at all, while strain AE1 grew after a period of about two weeks. From this plate a second strain, AE2, was isolated which had the same properties as strain AE1 except that it grew spontaneously with phenacetin as single carbon and energy source. These observations were reproducible. Strain ATCC15668 had no amidase activity which could be induced by phenacetin.

Induction

The average amidase activity (substrate: *p*-nitroacetanilide) of the crude extract from uninduced bacteria was $0.16 \mu\text{mol/min/mg}$ (25 experiments). The extremes were 0.05 and $0.51 \mu\text{mol/min/mg}$. Repeated measurements showed a large scattering of the values: the relative standard deviation was 75 % of the mean. Phenacetin was the best inducer (Figs. 1 and 2 and Table 1). In some tests acetamide, xylocaine, acetanilide and aniline showed an induction too (Tables 1 and 2), but the induction with phenacetin was more intensive and reproducible.

Fig. 1. Total amidase activity as a function of the phenacetin concentration. Points indicate the mean values of 15 experiments, vertical bars represent the standard deviation.

Fig. 2. Specific activity of the supernatant liquid from lysed bacteria as a function of the phenacetin concentration of the growth medium. Points indicate the mean values of 15 experiments, vertical bars represent the standard deviation.

Growth of bacteria

We tested 0.5 % (w/v) succinate, *p*-hydroxybenzoate, lactate and adipate as carbon sources. Growth was best with lactate or succinate, and lactate was therefore chosen as the standard carbon and energy source. The lactate concentration optimal for the induction was dependent on the phenacetin concentration, the highest activity being obtained with 0.22 % lactate at a phenacetin concentration of 0.1 %. Higher lactate concentrations increased growth but

Table 1. *Induction of the amidase from P. acidovorans* AEI

The values are the results of individual experiments. They represent the relative increase of the specific activity as compared with controls without the inducer. Twenty-five other compounds were also tested (see Methods) but none had a significant inducing effect.

Concn of the inducer (%)...	Increase in specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	
	0.01	0.03
Acetanilide	1.16, 2.42, 3.19	34.6
Acetamide	3.88, 1.35	1.36, 1.47
Aniline	1.56, 1.78	1.49
Xylocaine	2.02, 1.07, 1.65	1.1
Phenacetin	6.63, 7.02, 2.62	47.2, 34.6

Table 2. *Effect of acetanilide concentration on amidase activity*

Acetanilide concn (% w/v)	0	0.03	0.04	0.06	0.08	0.1
Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	0.029	0.225	0.145	0.212	0.80	0*
Induction factor	1.0	7.7	5.0	7.26	27.4	
Total activity/l medium ($\mu\text{mol}/\text{min}$)	4.0	37.0	10.7	16.7	15.7	

* No measurable growth.

Table 3. *Bacterial growth with different phenacetin concentrations*

The lactate concentration was 0.22 % (w/v). Bacteria were grown for 72 h at 30 °C.

Phenacetin (1 %, w/v)	$10^{-8} \times$ Total counts (bacteria/ml)	$10^{-8} \times$ Viable counts (bacteria/ml)
—	20	10.5
0.02	19	9.8
0.06	15	7.8
0.10	6	5.1
0.14	3	3.6

inhibited the induction, presumably due to 'catabolite repression' (Magasanik, 1961). Although higher concentrations of phenacetin inhibited bacterial growth (Table 3), induction of enzyme activity was enhanced even when some of the phenacetin remained undissolved after autoclaving. After 72 h growth, undissolved phenacetin could no longer be detected. The lag phase of the growth curve was prolonged by the addition of phenacetin. With 0.1 % phenacetin the early exponential phase began after 20 h. Maximal growth yield was then reached after about three days instead of one day.

Disruption of bacteria

The bacteria could successfully be disrupted by ultrasonic treatment. A high protein yield in the supernatant liquid could be reached after 4 min of sonication and was not much higher after 6 min of treatment. However, a serious disadvantage of this method was the destruction of enzymic activity. Bacterial disintegration with lysozyme in the presence of EDTA proved to be a better method for the conservation of enzyme activity. A concentration of 0.025 M-EDTA was found to be optimal for the yield of solubilized protein, and for activity and specific activity (Table 4). Some of the bacteria lysed with EDTA alone, i.e. in the absence of lysozyme, particularly if the bacteria had previously been frozen. The lysed bacteria were

Table 4. *Lysis of P. acidovorans* AEI by lysozyme in the presence of different EDTA concentrations

After incubating with lysozyme for 2 h at 30 °C the cultures were centrifuged for 30 min at 100000 g. The protein concentration and amidase activity of the supernatant fluid were determined; the values are the results of two separate experiments.

EDTA concn (mol/l)	Protein (mg/g bacteria)		Enzymic activity ($\mu\text{mol/min/g}$ bacteria)		Specific activity ($\mu\text{mol/min/mg}$ protein)	
—	26.8	24.6	184	154	6.89	6.24
0.0063	38.2	37.6	184	194	4.83	5.18
0.0125	35.5	31.5	234	228	6.55	7.23
0.025	46.8	43.0	607	650	13.0	15.2
0.05	39.1	38.5	391	481	10.0	12.4

Table 5. *Purification of a p-nitroacetanilide hydrolysing enzyme from P. acidovorans* AEI

This preparation was carried out with 63 g (wet wt) bacteria.

Step	Volume (ml)	Protein concn (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol/min/mg}$)	Total activity ($\mu\text{mol/min}$)	Purification factor	Yield (%)
Crude extract (lysis + washing of sediment)	448	11.3	5060	10.4	52600	1	100
Addition of streptomycin	463	9.4	4340	11.5	50000	1.2	95
Ammonium sulphate fractionation	41	86	3530	14.3	50500	1.4	96
Desalting by gel filtration (Sephadex G-50)	236	15	3540	14.5	51400	1.4	97.7
DEAE-Sephadex A-50	208	3.3	683	64	43700	6.2	83
Gel filtration (Sephadex G-100)	70	5	350	88.4	30900	8.5	58.7
Hydroxyapatite	116	1.2	134	131.5	17600	12.7	33.4

extremely viscous, probably because of the liberation of high molecular weight nucleic acids. The viscosity disappeared after precipitation with streptomycin sulphate. A reproducible solubilization procedure was of particular importance for the induction experiments. The most reproducible method was lysis by lysozyme. If uninduced bacteria of one batch were divided into several portions and lysed, the standard deviation of the specific activity was only $\pm 6\%$, the standard deviation of protein yield was $\pm 7\%$, and of the total activity $\pm 6.5\%$ of the respective average.

Isolation of the enzyme

Each preparation started from 50 to 70 g (wet wt) bacteria. An example of the purification procedure of the enzyme is given in Table 5. The total purification is 12.7-fold and the yield 33%. The purification was not very impressive because in the crude extract the enzyme is already the predominant protein. The individual purification steps may be summarized as follows:

Step 1: lysis of the bacteria and solubilization of the enzyme. The lysed bacteria were highly viscous. To obtain a clear extract the preparation had to be centrifuged at 100000 g for 30 min. On average, the supernatant contained 46 ± 9.3 mg protein/g lysed bacteria. The specific activity was 9.3 ± 1.7 $\mu\text{mol/min/mg}$ protein. The process of washing liberated an additional 25.5 ± 6.15 mg protein/g bacteria with an average specific activity of 7.0 ± 1.8 $\mu\text{mol/min/mg}$. Altogether, more than 75% of the total activity was recovered in the super-

Fig. 3. Fractional precipitation by ammonium sulphate. The total activity before the addition of ammonium sulphate is taken as 100 %. Each point represents the mean of four experiments.

nant liquid (100 % activity was determined with the suspension of lysed bacteria before centrifugation). Intact bacteria had only half of the total activity.

Step 2: removal of nucleic acids. Relatively low phosphate and EDTA concentrations, e.g. 0.025 M-phosphate buffer with 0.00125 M-EDTA, were essential to precipitate nucleic acids by streptomycin. Otherwise the precipitate immediately redissolved and reappeared on dilution with distilled water. The sediment of lysed bacteria was therefore washed with distilled water instead of buffer. Before removal of nucleic acids the E_{260} to E_{280} ratio was about 1:4; after streptomycin precipitation it had dropped to 1:1.

Step 3: ammonium sulphate fractionation. Unlike the crude extract, the purified enzyme was rather susceptible to ammonium sulphate and could not be precipitated without considerable loss of activity. The fractional precipitation of the amidase by stepwise addition of $(\text{NH}_4)_2\text{SO}_4$ is shown in Fig. 3. Half of the total activity was precipitated at 52 % ammonium sulphate saturation. For the enzyme isolation the fractionation of the protein was carried out in two steps, 0 to 30 % saturation and 30 to 65 % saturation. The sediment of the second step contained 90 ± 11 % of the activity. However, the purification was 1.3 ± 0.12 -fold only. The specific activity after ammonium sulphate fractionation was 11.5 ± 2.5 $\mu\text{mol}/\text{min}/\text{mg}$.

Separation by column chromatography

Step 4: DEAE-Sephadex A-50. The enzyme was completely adsorbed to the gel. The enzyme was eluted at a NaCl concentration of 0.18 ± 0.02 M (Fig. 4). The purification was 4.4 ± 0.4 -fold, and the specific activity after this step was about 60 $\mu\text{mol}/\text{min}/\text{mg}$.

Step 5: gel filtration. Of several gels tested the best separation was achieved with Sephadex G-100 (Fig. 5). Moreover, by gel filtration the enzyme was desalted and therefore could immediately be applied to the hydroxapatite column. The purification was 1.6 ± 0.2 -fold. This was in good agreement with the fact that about 50 % of the applied protein mixture already consisted of the enzyme. The yield was unusually low in this step (65 ± 8.5 %), probably because the enzyme became increasingly unstable with increasing purification.

Fig. 4. Column chromatography of amidase on DEAE-Sephadex A-50. The protein was eluted with a linear gradient of from 0 to 0.35 M-NaCl. The gradient was measured by flame photometry. —, E_{280} ; ----, gradient; ·····, amidase activity.

Fig. 5. Gel filtration of amidase on Sephadex G-100 (after chromatography on DEAE-Sephadex A-50). —, E_{280} ; ·····, amidase activity.

Fig. 6. Chromatography of amidase on hydroxyapatite. Gradient elution. The phosphate gradient was measured by conductometry. —, E_{280} ; ---, gradient; ·····, amidase activity.

Step 6: adsorption chromatography with hydroxyapatite. Preliminary experiments showed that the enzyme, if dissolved in 0.005 M-phosphate buffer pH 8.0, was completely adsorbed to the hydroxyapatite. The elution, however, raised problems. Stepwise elution with phosphate buffer of increasing concentrations yielded one amidase peak with every elution step, whereas on gradient elution, only one peak trailing on the descending part (Fig. 6) or occasionally two peaks were obtained. Multiple enzyme peaks did not necessarily indicate heterogeneity but were probably artefacts (Tiselius, Hjerten & Levin, 1956). To investigate this possibility further, enzyme-containing peaks from several experiments in which two peaks had been obtained were examined separately. After re-chromatography the elution pattern was the same for both peaks and corresponded to the pattern of the first chromatography. The enzyme from both peaks had the same molecular weight as determined by gel filtration. Analytical disc electrophoresis in 6% polyacrylamide gel revealed the same electrophoretic mobility for the amidase of either peak. In addition, both forms were inhibited by diethyl-*p*-nitrophenyl phosphate. These results indicate that the two forms were identical.

By chromatography on hydroxyapatite the final impurities could be removed, provided that specific activity before chromatography was 100 $\mu\text{mol/min/mg}$ or more. The average yield was 72% and the specific activity $131 \pm 5.3 \mu\text{mol/min/mg}$ (5 preparations). The chromatography with hydroxyapatite was reproducible only if all conditions, including temperature and velocity of elution, were kept constant.

Stability of the enzyme

In the presence of organic solvents and at pH values below 6.5 or above 9.5, enzyme denaturation occurred rapidly. Precipitation of the enzyme by acetone, methanol or ethanol at temperatures below 0 °C led to complete inactivation. The stability in tris buffer and phosphate buffer was the same. Glycerol (2%, v/v) stabilized the enzyme, whereas a sample with 0.001 M-mercaptoethanol showed less stability. The presence of glycerol in tris buffer resulted in a 20% higher residual activity after 20 days. The stability of the enzyme decreased

Fig. 7. Analytical disc electrophoresis of purified enzyme in 6 % polyacrylamide gel according to Hedrick & Smith (1968). Above: Amidoblack stain for proteins. Below: substrate stain (α -naphthyl acetate) for esterase activity.

with increasing purity and at higher dilutions. Chromatographic procedures performed with pure enzyme caused up to 90 % loss of activity. After storing three months at -18°C , 77 % of the activity of the highly purified enzyme was recovered.

Criteria of purity

Analytical disc electrophoresis. Disc electrophoresis of the crude extract from induced bacteria showed, among a variety of protein bands, one prevailing band which could be stained by α -naphthylacetate. Enzyme preparations with a specific activity of $125\ \mu\text{mol}/\text{min}/\text{mg}$ were homogeneous on disc electrophoresis (Fig. 7). The enzyme band had the same electrophoretic mobility as the main band of the crude extract and also reacted with α -naphthylacetate.

*Titration with diethyl-*p*-nitrophenyl phosphate.* The enzyme was strongly inhibited by diethyl-*p*-nitrophenyl phosphate (Alt, Heymann & Krisch, 1975). The highest specific activity, which was achieved in three out of eight preparations, was $137\ \mu\text{mol}/\text{min}/\text{mg}$. Only with these preparations was the equivalent weight identical with the molecular weight as determined by gel filtration and with the chain weight as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (Alt *et al.* 1975).

DISCUSSION

Enzymes hydrolysing amides of low molecular weight are quite common, not only in micro-organisms (Betz & Clarke, 1973; Kelly & Clarke, 1962; Myers, Tol & DeJonge, 1957; Draper, 1967) but also in mammalian tissues. In many cases they are unspecific carboxylesterases (EC. 3.1.1.1), e.g. the esterase from pig liver which hydrolyses acetanilide as well as other aromatic amides and esters (for a review see Krisch, 1971). The mammalian enzymes are localized preferentially in the microsomal fraction, while the amidase of *Pseudomonas acidovorans* AE1 presumably occurs in the cytoplasm or may be loosely bound to membranes.

The high initial activity after induction, the fast growth of *P. acidovorans* AEI as well as the relatively convenient isolation procedure make this amidase a suitable object for a closer examination of its catalytic and molecular properties. The further characterization of the enzyme and its active site are reported elsewhere (Alt *et al.* 1975). Catabolite repression has also been observed with the acetamide hydrolysing amidase of *Pseudomonas aeruginosa* 8602/A (Brammar & Clarke, 1964). However, with the enzyme of *P. acidovorans* AEI this cannot be overcome simply by adding more inducer, because phenacetin or a metabolite of it proved to be toxic for bacterial growth. For this reason it is important to keep the lactate concentration as low as possible.

The physiological function of mammalian esterases and amidases has not yet been elucidated. The liver esterase is probably important for the hydrolysis of certain drugs like acetanilide, phenacetin or procaine, and thus could contribute to the so-called detoxication of foreign compounds. The function of amidases or esterases in bacteria may be more easily explained. Most of them are inducible by substrates or analogous substances, for example, the enzymes of *Mycobacterium smegmatis* (Diaper, 1967) and of *P. aeruginosa* (Kelly & Clarke, 1962; Betz & Clarke, 1973). It can be assumed that these inducible enzymes are functioning in the dissimilation of carbon and nitrogen sources.

The aliphatic amidase from *P. aeruginosa*, which preferentially hydrolyses short-chain aliphatic amides, has been extensively studied by Clarke and her collaborators. Recently, mutants grown on acetanilide as sole carbon source were isolated. They produced amidases with different substrate specificities and electrophoretic properties from the parent strain (Brown & Clarke, 1972). In another comparative study the biochemical and immunological properties of amidases from various *Pseudomonas* species were investigated. The amidases in extracts of cultures of acetamide-grown *P. acidovorans* strains 14 and NCIB9681 showed strong cross-reactivity with an antiserum against *P. aeruginosa* A amidase (Clarke, 1972). They are possibly identical with the acetamide splitting activity found by us in crude extracts from *P. acidovorans* AEI. This activity, however, was lost during the purification procedure and the highly purified enzyme does not hydrolyse acetamide (Alt *et al.* 1975).

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