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The expression in *E. coli* of a polymeric gene coding for an esterase mimic catalyzing the hydrolysis of *p*-nitrophenyl esters

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We have prepared a hybrid protein consisting of seven esterase units, Glu-Ala-His-Ala-Ser-Phe-Phe-Phe, fused to the N-terminal of galactokinase *(E. coli)*. The structural gene for this bifunctional protein was obtained by cloning a polymer made up of three chemically synthesized oligonucleotides to which the galactokinase gene was fused in frame. The hybrid protein was purified to homogeneity with the aid of the galactokinase moiety and showed an M_r of 51 000–53 000. The preparation could catalyze the hydrolysis of *p*-nitrophenyl esters and, due to the inbuilt hydrophobic spacers, Phe-Phe-Phe, improved catalysis of more hydrophobic substrates was obtained.

Synthetic enzyme; Esterase; Galactokinase; Gene fusion; *(E. coli)*

1. INTRODUCTION

There has been considerable interest over the years in obtaining preparations mimicking enzymes, notably of proteases, since the rules governing enzyme catalysis are known for many of these enzymes. One line of approach in this direction has been to synthesize by chemical means oligo- and polypeptides consisting of the amino acids believed to participate in hydrolytic reactions [1,2]. In more elaborate approaches a 'substrate-binding pocket' has been built or utilized, close to or around which the catalytic groups are arranged. Examples of the latter include the use of a solid matrix such as Sephadex [3], cyclodextrins [4,5] or 'spherands' [6].

Here, we have applied genetic engineering techniques to examine the possibility of cloning synthetic repeating genes to a peptide expressing hydrolytic activity. We chose to prepare and clone a gene coding for multiple repeats of the octapeptide, Glu-Ala-His-Ala-Ser-Phe-Phe-Phe, which contain the catalytic groups necessary for ester hydrolysis. The structural gene of galactokinase (*E. coli*), *galK*, was fused in frame with the synthetic esterase gene. When this chimaeric gene was expressed in *E. coli*, the galactokinase moiety considerably simplified isolation and purification. The hybrid enzyme obtained, esterase-galactokinase, was purified to homogeneity and shown to be hydrolytically active against activated esters. Most likely due to the Phe-Phe-Phe moieties between the catalytic groups, the synthetic esterase showed a higher relative catalytic activity to more hydrophobic substrates as compared with monohistidine or the histidine-containing oligopeptide Ser-His-Asp.

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2. EXPERIMENTAL

2.1. *Bacteria and plasmids*

E. coli C600K$^-$ (galE$^+$T$^+$K$^-$, lac$^-$, thr$^-$, leu$^-$),

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obtained from K. McKenney, was used as a recipient for bacterial transformation. The following plasmids were used in this study: pBR328 [7], pUC9 [8] and pzk5 [9].

2.2. *Chemicals*

[^{14}C]Galactose for galactokinase assay and [^{35}S]-thio-dATP for DNA sequencing were obtained from Amersham and New England Nuclear, respectively. The oligopeptide Ser-His-Asp was purchased from Serva. All other chemicals were from Sigma.

2.3. *Enzymes*

Restriction endonucleases and enzymes used in DNA cloning were obtained from Boehringer Mannheim. Enzyme reactions were buffered as recommended by the manufacturer and performed as described [10]. Galactokinase (*E. coli*) was purified according to [11].

2.4. *Construction of the esterase gene*

Three chemically synthesized oligonucleotides (purchased from KabiGen, Stockholm) d(GpAp-ApGpCpGpCpApCpGpCpGpApGpCpTpTpTpTp-TpCpTpTpT) (no. 1), d(CpGpCpGpTpGpCpGp-CpTpTpCpApApApGpApApApApApGpCpT) (no. 2) and d(CpGpCpGpTpGpCpGpCpTpTpCp-TpGpCpA) (no. 3), were used to build up the esterase gene. Each oligonucleotide (1 μg) was phosphorylated in a 10 μl reaction mixture with T_4 polynucleotide kinase. After 60 min at 37°C the three oligonucleotides were mixed in a 100:100:1 ratio of no. 1:no. 2:no. 3, heated to 60°C for 5 min and then slowly cooled to room temperture to allow self-hybridization. T_4 DNA ligase was added and the mixture incubated overnight at room temperature. The polymers were concentrated by ethanol precipitation. Finally, approx. 0.5 μg of the polymer mixture was treated with the Klenow fragment for 15 min to produce DNA fragments with a *Pst*I site at one end and a blunt end at the other (fig.1). This mixture was directly ligated to pBR328 previously digested with *Pst*I and *Pvu*II. The nucleotide sequences of a few of these DNA fragments were confirmed by the Sanger dideoxy chain termination method [12]. The esterase genes were transferred from pBR328 to pUC9 as *Pst*I/*Eco*RI fragments. To one of the plasmids obtained, pES7, carrying seven esterase units, the *galK* gene was joined as an *Eco*RI fragment to the 3′-end of the esterase gene. The resulting plasmid, pES27, codes for an in-frame fusion between the esterase and the galactokinase. The small fragment (100 bp) between the *Pvu*II and *Eco*RI sites in pBR328 was used as a linker region between the two catalytic entities (fig.2).

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          Glu  Ala  His  Ala  Ser  Phe Phe Phe
5'        GAA GCG CAC GCG AGC TTT TTC TTT GAA GCG CAC GCG   3'
3'  A CGT CTT CGC GTG CGC TCG AAA AAG AAA CTT CGC GTG CGC   5'
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Fig.1. Nucleotide sequence of a segment of the cloned esterase gene, the catalytic unit, showing how it is constructed from three chemically synthesized oligonucleotides.

2.5. *Enzyme assays and protein determination*

Galactokinase activity was determined using [^{14}C]galactose [13]. One unit of enzyme phosphorylates 1 μmol galactose per min at 30°C. Esterase activity was assayed by following the

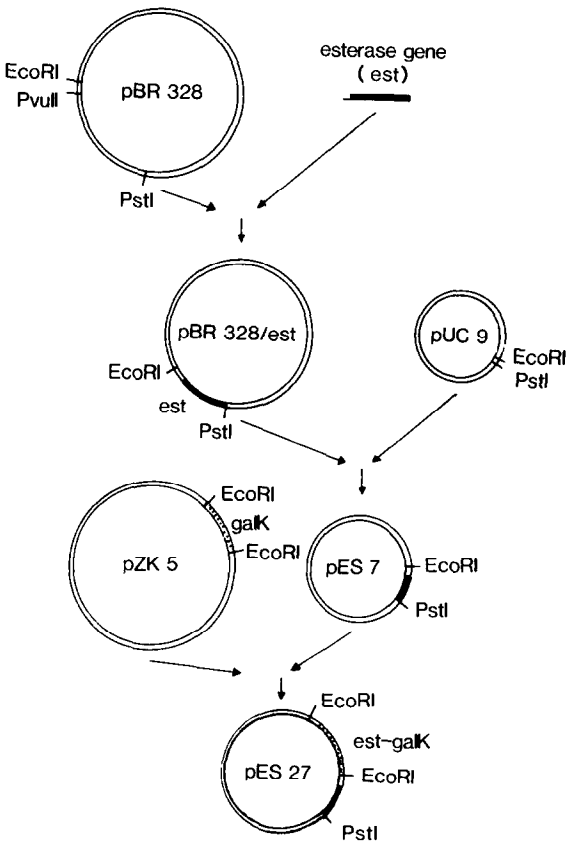

Fig.2. Construction of the esterase-galactokinase fusion vector.

hydrolysis of *p*-nitrophenyl esters spectrophotometrically at 405 nm. The substrates, *p*-nitrophenyl acetate, propionate, butyrate, caproate and caprylate, were dissolved in acetonitrile. Subsequently, ethanol and 0.07 M potassium phosphate buffer, pH 7.8, were added to a final composition of solution of 1:4:95 of acetonitrile/ethanol/buffer, respectively. All measurements were carried out under first-order reaction conditions, i.e. with the catalyst in excess of ester. Substrate and catalyst concentrations were 0.1 and 0.2 mM, respectively.

During protein purification *p*-nitrophenyl butyrate was used as substrate. This compound showed greater stability in the presence of interfering enzymes in an *E. coli* homogenate as compared to *p*-nitrophenyl acetate. Thus, 1 unit of esterase was defined as a change of one *A* unit/min at 21–22°C using *p*-nitrophenyl butyrate as substrate.

Protein concentrations were determined using the method of Bradford [14].

2.6. *Protein purification*

All operations were performed at 0–4°C.

2.6.1. Preparation of extract

The hybrid protein was purified from *E. coli* C600K$^-$ carrying pES27 grown to middle exponential phase ($A_{550} = 2.5$) in LB medium containing 100 mg/l of ampicillin. The cells were harvested by centrifugation at 9000 × *g* and washed once with chilled 0.005 M sodium phosphate buffer at pH 7.0. Ordinarily 1 l yielded 4–5 g packed cells.

The cells were suspended in 4 vols of 0.02 M sodium phosphate buffer containing 10 mM mercaptoacetic acid and 1 mM EDTA at a final pH of 7.0 and then sonicated for 5 min in a sonic oscillator (B-30 sonifier, Branson Sonic Power). The suspension was clarified by centrifugation at 20000 × *g* for 10 min.

2.6.2. Ammonium sulfate precipitation

The suspension was made 50% saturated with respect to ammonium sulfate by slow addition of solid $(NH_4)_2SO_4$. After 30 min the precipitate was removed by centrifugation at 20000 × *g* for 30 min and suspended in a minimum volume of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 mM mercaptoacetic acid (buffer A).

2.6.3. Sephacryl S-400 superfine gel filtration

The protein solution was subjected to chromatography on a Sephacryl S-400 SF column (97 × 2.5 cm) equilibrated with buffer A. Fractions containing both galactokinase and esterase activities were concentrated by ammonium sulfate precipitation. The sample was dissolved in 0.02 M sodium phosphate, pH 7.4, containing 1 mM EDTA and 10 mM mercaptoacetic acid (buffer B) and dialyzed against the same buffer.

2.6.4. DEAE-Sephacel fast flow

The sample was clarified by centrifugation for 30 min at 20000 × *g* and the supernatant applied to DEAE-Sephacel previously equilibrated with buffer B. The column was washed with 5–10 vols of the same buffer and the hybrid protein eluted with a linear gradient of 0–300 mM NaCl.

2.6.5. Phenyl-Sepharose

NaCl was added to the esterase-galactokinase-containing fractions to a final concentration of 2 M. The sample was adsorbed on a phenyl-Sepharose column (2 ml) and washed with 10 vols buffer B containing 2 M NaCl and then with 10 vols buffer B containing 0.5 M NaCl. The hybrid protein was eluted with water.

3. RESULTS

3.1. *Purification of hybrid protein*

During initial work on purification of the synthetic esterase it was found that an essential prerequisite for successful isolation of the artificial polypeptide was the fusion to a 'marker' enzyme which also could serve as an 'affinity tail' during protein purification. In addition, the M_r was increased which is highly favourable since there were several low-M_r proteins exhibiting esterase activity and thus 'masking' the esterase mimic during, e.g. gel filtration. Galactokinase has been used for gene fusion purposes earlier [9,18]. It is a small monomeric enzyme (M_r 40000) and shows a low intrinsic esterase activity. Thus, by taking advantage of both enzymic activities we were able to purify the hybrid protein using conventional chromatographic procedures: ammonium sulfate precipitation, gel filtration (fig.3), ion-exchange (fig.4) and hydrophobic chromatography.

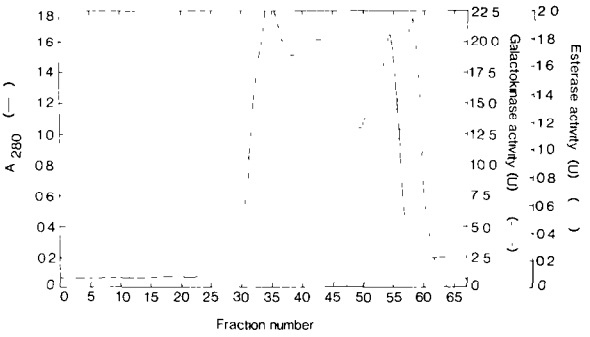

Fig.3. Fractionation of esterase-galactokinase on Sephacryl S-400 SF (flow rate, 0.33 ml/min; fraction volume, 6.6 ml).

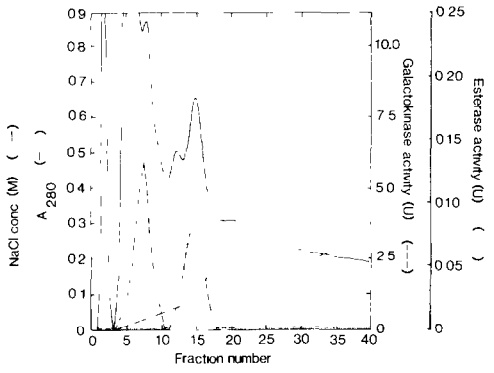

Fig.4. Fractionation of esterase-galactokinase on DEAE-Sephacel (flow rate, 0.43 ml/min; fraction volume, 8.6 ml).

As judged from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis this resulted in a homogeneous preparation (fig.5). According to SDS-PAGE and gel filtration the protein was monomeric since both analyses indicated an M_r of 51 000–53 000. This was in agreement with the theoretically calculated value of M_r 52 000. The esterase-galactokinase fusion protein showed specific activities of 60 and 0.45 U/mg for the galactokinase and esterase moieties, respectively.

3.2. *Catalytic properties of the hybrid protein*

The catalytic properties of the bifunctional enzyme were evaluated by determining the rate of hydrolysis of *p*-nitrophenyl esters of acetic, pro-

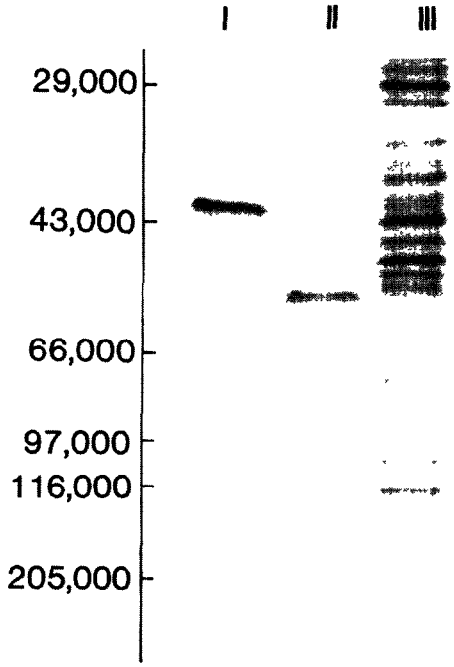

Fig.5. SDS-polyacrylamide gel electrophoresis of the esterase-galactokinase fusion protein on a 10% acrylamide gel. (I) Purified native galactokinase. (II) Purified esterase-galactokinase. (III) Crude extract of *E. coli* C600K$^-$ carrying pES27.

pionic, butyric, caproic and caprylic acid. The observed rate constants were compared with those of naturally occurring esterases. Table 1 gives the relative rate of hydrolysis of *p*-nitrophenol acetate for a variety of catalysts, both amino acids and peptides, where an equimolar concentration of each esterase was used. The catalytic activity of histidine has been used as reference. The data presented suggest that there is a cooperative effect between the functional groups of an oligo- or polypeptide compared with free amino acids since the latter, alone or in a mixture with other free amino acids, show a much lower relative activity.

To evaluate the influence of the galactokinase part of the hybrid enzyme on the esterase activity, the purified enzyme was subjected to proteolytic degradation by trypsin immobilized on tresyl chloride-activated Sepharose 4B [15]. This resulted in a total degradation of both the galactokinase and the spacer but left the esterase intact since the latter has no lysine or arginine residue. The catalytic capacity was reduced by 9% suggesting

Table 1

Hydrolysis of *p*-nitrophenyl acetate

Catalyst	Relative catalytic activity
Histidine (His)	1.0
Glutamic acid (Glu)	0.02
Aspartic acid (Asp)	0.004
Serine (Ser)	0.05
Ser + His + Asp	1.6
Ser-His-Asp	3.0
Esterase-galactokinase	3200
Galactokinase	46
Chymotrypsin	2300[a]
Papain	230 000

[a] *p*-Nitrophenyl acetate is not a suitable substrate for chymotrypsin. An appropriate substrate, such as benzoyl-L-tyrosine ethyl ester, gives a value corresponding to 300 000

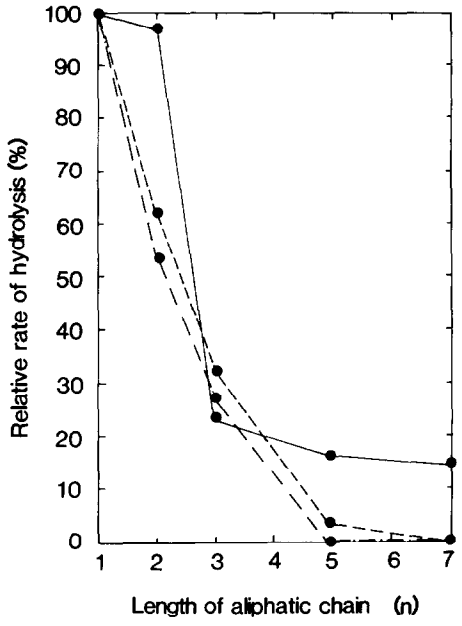

Fig.6. Relative ratio of hydrolysis of *p*-nitrophenol esters of acetic acid ($n = 1$), propionic acid ($n = 2$), butyric acid ($n = 3$), caproic acid ($n = 5$) and caprylic acid ($n = 7$) using different catalysts: histidine (·····), Ser-His-Asp (– – –) and esterase-galactokinase (——).

that the effect of the galactokinase moiety on the esterase was negligible. In addition, native galactokinase as such showed a low intrinsic esterase activity.

To examine the effect of the Phe-Phe-Phe groups which were introduced as substrate-binding sites in analogy to Nilsson and Mosbach [3], the catalytic activity was tested on *p*-nitrophenyl esters of carboxylic acids with different lengths of the aliphatic chain. As can be seen from fig.6 the esterase exhibited a higher relative catalytic activity against more hydrophobic substrates as compared with mono-histidine or Ser-His-Asp, thus indicating that the hydrophobic substrates interacted more favourably with the hybrid protein.

4. DISCUSSION

In this study we have made an attempt to design an enzyme-like catalyst from the 'ground up' [16]. We have chosen to prepare an esterase/protease mimic since the catalytic mechanism is known for many of these hydrolytic enzymes. When studying naturally occurring enzymes, the polypeptide chain might be looked upon as being composed of a substrate-binding site and a catalytic site. Therefore, it seems as an essential feature of an enzyme designed de novo to have both of these sites in a final construction. Starting with the catalytic site, ester hydrolysis can be based upon monofunctional catalysis using imidazolyl groups, but much improved catalysis is obtained if different active groups cooperate. Furthermore, these groups should have an optimal internal environment. However, it is not possible to predict the tertiary structure of a protein from its amino acid sequence alone and thus a prediction on the catalytic efficiency is precluded. Nevertheless, by creating a long flexible polypeptide chain carrying several repetitive catalytic groups some of them might interact suitably for catalysis by a random folding process. Glutamic acid was chosen instead of aspartic acid to give increased flexibility to the polypeptide and to permit the catalytic groups of the artificial esterase to interact with each other in a similar mode to the 'charge-relay system' of chymotrypsin [17]. When the catalytic efficiency of the esterase is compared with, e.g. Ser-His-Asp, it seems plausible that such favourable interactions at least take place in part, but the individual role

of these functional groups has to be evaluated by, e.g. selective chemical modification. This comparison is based only on the release of *p*-nitrophenol in the ester hydrolysis described. The deacylation step, necessary for turnover, has not been evaluated. A detailed kinetic analysis will be carried out later.

In addition to the catalytic activity of the polypeptide, the substrate-binding region is important. Since the conformation of the artificial polypeptide is unknown, it is difficult to design a binding site, but by simply introducing hydrophobic regions, Phe-Phe-Phe, in between the catalytic groups a stronger affinity for hydrophobic substrates was obtained as compared to monohistidine or Ser-His-Asp.

Since knowledge of protein folding and enzyme mechanisms continues to advance, more intelligent conclusions can be drawn as to how to construct improved synthetic catalysts employing, e.g. computer graphics. The use of genetic engineering techniques to verify such theoretical assumptions is highly advantageous because of the ease with which synthetic oligonucleotides are made and subsequently expressed in large amounts in microorganisms. The synthetic esterase described catalyzes only hydrolysis of activated esters and as such is to be considered only as a first step towards the preparation of artificial enzyme-like catalysts. One of the main purposes of this study has been to focus attention on the genetic engineering approach as an alternative to the previously described purely chemical procedures. We are convinced that artificial enzyme-like catalysts produced by biological means will find widespread use in many areas in the future. Certainly, practical applications will be found in bioorganic synthesis of esters by reversal of the ester hydrolysis described and in various bioanalytical procedures.

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