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Extraction and Assay
of Escherichia coli Penicillinase
and Enzyme Immobilization
on Hydrophobic Supports

by

© Christin T. Choma (B.Sc., Hons.)

A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment of
the requirements for the degree of Master of
Science

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The undersigned hereby recommend to the Faculty of Graduate Studies acceptance of this thesis, entitled 'Extraction and assay of Escherichia coli penicillinase and enzyme immobilization on hydrophobic supports' submitted by Christin T. Choma (B.Sc., Hons), in partial fulfillment of the requirements for the degree of Master of Science.

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For Mom, Dad,
Liz and George

ABSTRACT

New methodologies for the assay and extraction of Escherichia coli periplasmic penicillinase are presented. The new penicillinase assay remedies the lack of specificity of the commonly used iodometric assays, while the extraction method is considerably simpler and faster than the conventional means of releasing periplasmic proteins from E. coli. Evidence is presented that the new extraction procedure efficiently extracts only periplasmic proteins, without releasing cytoplasmic proteins. This simple extraction method not only facilitates the assay of penicillinase in a bacterial culture, but will also be useful for large-scale purification of periplasmic penicillinase and other periplasmic proteins.

While investigating the specificity of the extraction method, it was noticed that penicillinase bound tightly to octyl-agarose, a hydrophobic protein fractionation material, to yield a very active immobilized enzyme preparation. The generality of this novel immobilization procedure was investigated by measuring the activity of various enzymes bound to hydrophobic supports. Most of the immobilized enzyme preparations were quite stable, suggesting that hydrophobic supports could be used for the industrial immobilization of enzymes. Therefore, several

attempts were made to synthesize inexpensive hydrophobic derivatives of cellulose to use as enzyme supports in industrial reactors.

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ABBREVIATIONS

A_{500}	absorbance at 500 nm
5'-AMP	adenosine 5'-monophosphate
Ap ^r	ampicillin resistance
AU	absorbance unit
BAEE	N-benzyl-L-arginine ethyl ester
BAN	benzoyl-DL-arginine p-nitroanilide
B.S.A.	bovine serum albumin
°C	degree Celsius
^{14}C	carbon-14
cAMP	adenosine 3',5'-cyclic monophosphate
cm	centimetre
cpm	counts per minute
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DMS·Na	methylsulphonyl carbanion
DMSO	dimethylsulphoxide
DNS	3,5-dinitrosalicylic acid
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediaminetetraacetate
i.d.	inside diameter
<u>lacI</u> -	lactose constitutive mutant
<u>leu</u>	requires leucine for growth
M	molar
mA	milliampere
μCi	microcurie

μg	microgram
<u>met</u>	requires methionine for growth
mg	milligram
min	minute
μL	microlitre
mL	millilitre
nm	nanometre
ONPG	O-nitrophenol- β -D-galactopyranoside
pABA	p-aminobenzamidine
PBE 94	Polybuffer exchanger 94
PEA	phenethyl alcohol
pI	isoelectric point
$^{32}\text{P}_i$	$[^{32}\text{P}_i]$ orthophosphate
R_f	relative flow
SDS	sodium dodecylsulphate
Tc^r	tetracycline resistance
TCA	trichloroacetic acid
<u>thi</u>	requires thiamine for growth
<u>thr</u>	requires threonine for growth
TMM	tris-maleate minimal medium
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume

GENERAL INTRODUCTION

Biotechnology offers a unique means of efficiently producing organic materials of practical value by controlling the activities of living cells, particularly microbes, or their enzymes. By this definition, biotechnology is not a new science. Aside from the ancient arts of brewing and baking, biotechnology has for decades been providing us with nutritional supplements such as amino acids and vitamins, and pharmaceuticals such as antibiotics and steroids. The further potential for controlling the activities of living cells is enormous, as the production of many organic compounds through the genetic manipulation of micro-organisms has recently become a reality. Although human insulin and growth hormone are the most widely publicized examples, other less known, although by no means less important examples, abound. An excellent introduction to Biotechnology can be found in a recent issue of 'Scientific American' (September, 1981), which is devoted to the subject.

Among the characteristics of biotechnology which make it so appealing in today's world is the possibility of regulating biological processes to make full use of renewable resources. Our carnage of the Earth's non-renewable resources has left us with few alternatives but to efficiently exploit biomass for our materialistic benefit. The most effective way of converting biomass

into practical compounds is through biological processes. Canada is very rich in biomass, and as such has the resources necessary to fuel a biotechnology industry. However, although the opportunities offered by biotechnology are presently being pursued in numerous areas of research and industry around the world, Canada's efforts in this field are just beginning. Still, the importance of biotechnology to Canada's future has been acknowledged by the Federal Government. In 1981, the Task Force on Biotechnology submitted its first report to the Minister of State for Science and Technology; several recommendations were made to stimulate biotechnology in this country.

The first Canadian biotechnology company, Bio-Logicals, was incorporated in 1979. Since then, several other companies have been established, notably Allelix in Toronto and Philom Bios in Saskatoon. Personnel with scientific experience in biotechnology will be required if these ventures are to succeed; therefore, universities have an obligation to provide instruction in biotechnology and related fields. When I first joined this laboratory to do my Master's degree, Dr. Yamazaki was in the process of shifting his research and teaching interests from purely academic topics to more applied, biotechnologically-oriented subjects. My thesis represents this laboratory's first attempts to be involved in biotechnological research.

One of the most exciting aspects of biotechnology is recombinant DNA technology, which is the production of hybrid DNA created by joining pieces of DNA from different sources. When the recombinant DNA is transferred to a micro-organism, the micro-organism may acquire novel genetic properties and the resultant ability to create a new product. An early example of the successful application of recombinant DNA technology was the 1978 report by Villa-Komaroff et al. that the rat proinsulin gene had been cloned into Escherichia coli. The gene product was expressed as a protein which could be easily modified to yield biologically-active insulin. The rat proinsulin gene was carried on a plasmid vector, pBR322, which exists in multiple copies in each bacterium bearing the plasmid. Plasmid pBR322 is a favourite cloning vehicle not only because it is multicopied, but also because it carries two easily recognized genetic markers, namely penicillin and tetracycline resistance. In fact, the proinsulin gene was inserted into the plasmid near the terminal portion of the β -lactamase, or penicillinase, gene. Thus, the hybrid gene product extracted from the bacterial clone exhibited both penicillinase activity and insulin affinity for anti-insulin antibody.

Like all bacterial proteins, penicillinase is synthesized within the cytoplasm. However, after synthesis, penicillinase is exported out of the cytoplasm and into the periplasmic space, an aqueous compartment

which surrounds the cytoplasm. The advantage of producing a hybrid, penicillinase-mammalian gene product is that the penicillinase portion of the protein protects cloned eukaryotic peptides from proteolysis within the cytoplasm by exporting the peptide across the bacterial inner membrane and into the periplasmic space. Furthermore, the degree of penicillinase activity in a bacterial culture reflects the concentration of the cloning vehicle on which the penicillinase and mammalian genes are carried.

If the production of cloned eukaryotic proteins by the hybrid method described above is to become commercially possible, a simple and quick method of extracting the hybrid penicillinase protein from large batches of bacteria will be needed. In addition, an efficient way of assaying penicillinase activity as a means of gauging the concentration of the gene product in the culture would also be useful. In Chapter 1 of this thesis, I present a penicillinase assay which permits the assay of enzyme in a bacterial culture not yet separated from its nutrient medium. This was not possible with previous penicillinase assay methods, as they were subject to interference from several components of growth medium. This attribute of the new penicillinase assay will be particularly useful in industry, where unnecessary purification of bacteria or protein serves only to escalate production costs. Also in Chapter 1, a very rapid means of extracting penicillinase from E. coli is

presented. This method is simpler than the more conventional process, and yet is equally efficient at extracting penicillinase. The possibility that the method extracts only periplasmic proteins from E. coli, without releasing cytoplasmic proteins, is examined in Chapter 2.

Another important aspect of biotechnology is immobilized enzyme technology. The purpose of immobilizing an enzyme upon an insoluble support is to allow enzyme and substrate to come into contact for a sufficient period of time for the desired reaction to take place, and then to be able to easily separate the product from the reusable enzyme. Because enzymes are specific in their catalytic properties, the product stream flowing from an enzyme reactor is relatively pure. For example, a single isomer in a complex substrate, or feed solution, can be selected by an immobilized enzyme and be converted into a single isomer of the product. Most reactions can be carried out under mild conditions of temperature and pressure. In addition, because the enzyme, which is usually of microbial origin, is reused many times, the cost of substrate to product conversion is kept low.

A number of immobilized enzyme processes have been commercialized. The major ways of immobilizing enzymes are through co-valent attachment to the enzyme support (or matrix), or through ionic interactions between enzyme and matrix. Although the first method permanently immobilizes enzymes, the harsh chemical conditions used during the

immobilization procedure sometimes cause protein denaturation; in addition, the rigid bonds formed between enzyme and matrix are not conducive to enzyme conformational changes during catalysis. Enzyme immobilization through ionic interactions is a much more gentle method, but enzyme leakage off the support is both a serious and common problem.

In Chapter 2, while investigating the specificity of our new extraction method for periplasmic proteins, I noticed that penicillinase bound strongly to a matrix which was substituted with long alkyl chains. As this substitution made the matrix very hydrophobic, penicillinase immobilization seemed to be occurring through hydrophobic interactions. The simplicity of producing this very active penicillinase preparation suggested that this could be a novel and gentle means of immobilizing enzymes in general. The activity and stability of several enzymes immobilized on hydrophobic supports is examined in Chapter 3.

The results of that investigation suggested that hydrophobic interactions could be used for the industrial immobilization of enzymes. Since the commercial hydrophobic matrices available for laboratory-scale studies would be prohibitively expensive for use on an industrial scale, Chapter 4 examines several attempts to produce inexpensive matrices by derivatizing cellulose with hydrophobic groups. The results obtained in the

course of these immobilization investigations have provided the framework for the development of other enzyme immobilization procedures in this laboratory.

MATERIALS, BACTERIAL STRAINS AND CULTURE CONDITIONS

Materials

Benzyl [^{14}C] penicillin (potassium salt) was obtained from the Amersham Radiochemical Centre; carrier-free ^{32}P , and ^{14}C -lysine from New England Nuclear; alumina thin-layer plates without fluorescent indicator, from Eastman Kodak Co.; octyl-agarose, dodecyl-agarose, Sephadex G-25, Polybuffer exchanger (PBE) 94 and Polybuffer 74 were all from Pharmacia Fine Chemicals; epichlorohydrin, bromododecane and other chemicals used in the syntheses described in Chapter 4 were all analytical grade and obtained from Aldrich Chemical Co.; benzylpenicillin (potassium salt) and all biochemicals used were from Sigma Chemical Co.; Bradford protein assay kit was from Bio-rad Laboratories; gel electrophoresis apparatus GE-2/4 and gel slab casting apparatus GSC-2 were from Pharmacia Fine Chemicals.

Bacterial Strains and Culture Conditions

The following E. coli strains were used in the course of this investigation: WA802 (met) and WA802/pBR322 (met, Ap^r , Tc^r) (provided by Dr. R. Watson, National Research Council, Ottawa); 490/pBR322 (met, thr, leu, Ap^r , Tc^r) (provided by Dr. D. Thomas, National Research Council, Ottawa); 3300 (lacI-) (provided by B. Bachman, E. coli

Genetic Stock Center, Yale University, Conn.); and CA8000
* (thi) (provided by Dr. J. Beckwith, Harvard Medical
School, Mass.).

Bacteria were grown by shaking at 37°C in Tris-maleate minimal medium (TMM) (Khan and Yamazaki, 1972), containing 0.4% (w/v) glucose as carbon source, 1 mM K_2HPO_4 , and supplemented with amino acids (50 µg/mL) and thiamine (10 µg/mL) where required. Cell cultures used in the investigations presented in Chapter 1 were grown in glucose, phosphate and 0.4% (w/v) salt-free casamino acids. Growth was followed by measuring absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 70 spectrophotometer using a round cuvette (11 mm i.d.). One absorbance unit (AU) in A_{500} · mL contains about 5×10^8 cells.

CHAPTER ONE ,

Simple assay and extraction of periplasmic penicillinase in Escherichia coli.

Introduction

A Gram-negative bacterium, such as E. coli, is composed of two compartments: the cytoplasm, which is bounded by the cytoplasmic or inner membrane, and the periplasm, which is situated between the inner and outer membranes. The outer membrane has a high protein content but relatively few protein species. Some proteins of the outer membrane function as pores through which small water-soluble molecules can diffuse, while others serve structural roles or are involved in the transport of specific substrates. The bacterium is protected from osmotic lysis by a rigid layer of peptidoglycan, a polysaccharide polymer cross-linked by short peptide bridges; the peptidoglycan is situated in the periplasmic space, adjacent to the outer membrane. The periplasmic space is an aqueous compartment containing a wide variety of protein species. Generally speaking, these proteins are either degradative enzymes or binding proteins involved in active transport of certain substrates.

Finally, there is the inner membrane, an area of intense enzymic activity which serves as the true permeability barrier of the cell.

Penicillinase, also known as β -lactamase or penicillin amido- β -lactamhydrolase (EC 3.5.2.6), is an enzyme which confers bacterial resistance to β -lactam antibiotics such as benzylpenicillin and ampicillin. The antibiotic effect of penicillins arises from their ability to block the last step in bacterial cell wall biosynthesis. Specifically, penicillins inhibit the transpeptidase which cross-links different peptidoglycan strands. Penicillinase inactivates these antibiotics by hydrolyzing the β -lactam bond (Fig. 1); this results in the production of the corresponding penicilloic acids, which have no antimicrobial effect.

Although the penicillinase gene is sometimes chromosomal (Lindstrom *et al.*, 1970), it is often coded for, by resistance transfer plasmids. Penicillinases in Gram-negative bacteria reside in the periplasmic space and are synthesized constitutively (Richmond and Sykes, 1973), while in the case of Gram-positive bacteria, penicillinases are excreted from the cell into the surrounding environment, and their synthesis is inducible. Several types of penicillinases from Gram-negative bacteria have been isolated, the most common being RTEM 1 (Matthew and Hedges, 1976). The periplasmic form of this enzyme is a single polypeptide of 263 amino acid residues.

Fig 1. Penicillinase hydrolysis of benzylpenicillin. The enzyme hydrolyzes the β -lactam bond to produce benzylpenicilloic acid, which has no antibiotic properties. The (*) signifies that the label of the $[^{14}\text{C}]$ benzylpenicillin used in this investigation resides in the benzyl ring.

Most antibiotic resistance genes, including penicillinase, are carried on small, extra-chromosomal, circular pieces of DNA called plasmids. Because they are self-replicating, relatively simple, and carry genetic markers such as antibiotic resistance, plasmids are often used in recombinant DNA experiments as acceptors of foreign DNA. Many of the plasmids found in Gram-negative bacteria are capable of being transferred from one bacterium to another by conjugation; these conjugative plasmids are also known as R factors. According to the National Institutes of Health, U.S.A., Recombinant DNA Guidelines, one of the most important safety features of a plasmid cloning vector is a low frequency of plasmid transmission. The plasmid pBR322 (molecular weight 2.6×10^6 daltons) has many characteristics which make it an excellent cloning vehicle: it has a very low frequency of transmission, carries two easily identified genetic markers (ampicillin and tetracycline resistance), exists in multiple copies within each bacterium and has convenient restriction sites for the insertion of foreign DNA (Bolivar et al., 1977). As explained in the General Introduction of this thesis, one of the most exciting cloning experiments performed to date involved the insertion of the rat proinsulin gene into the RTEM 1 penicillinase gene of pBR322 (Villa-Komaroff et al., 1978). As the mammalian peptide was exported to the periplasmic space, it did not suffer proteolysis.

Furthermore, since the number of protein species is fewer in the periplasmic space than in the cytoplasm, the exportation of the peptide facilitated its purification.

In view of the advantages of cloning hybrid penicillinase-eukaryotic proteins, a simple and accurate means of measuring penicillinase activity in a bacterial culture is important. Since penicillinase hydrolyzes the β -lactam bond of penicillins to produce penicilloic acid, the most common method for assaying penicillinase activity depends on the reduction of iodine by penicilloic acid but not by penicillin. Reaction velocities are estimated by measuring the rate of decolorization of a starch-iodide complex by penicilloic acid (Novick, 1962). However, the major disadvantage of this assay is the non-specific consumption of iodine by compounds other than the hydrolysis products of β -lactam antibiotics. Indeed, several amino acids, nutrient broths and even minimal bacterial growth media have been shown to consume considerable amounts of iodine (Lindstrom and Nordstrom, 1972). In this chapter, we present a penicillinase assay which is specific for the enzyme only.

Commercial production of mammalian proteins by the hybrid method discussed above will require an inexpensive, yet efficient means of extracting the hybrid protein from the bacterial periplasm. Previously, ultrasonication (Richmond, 1975) and osmotic shock (Neu, 1968) have been used to release periplasmic penicillinase. Efficiency and

reproducibility in ultrasonication critically depend on the performance of the probe. Since this performance tends to change with usage, the degree of cell disintegration needs to be monitored by an independent method. Furthermore, since both cytoplasmic and periplasmic proteins are released by ultrasonication, the purification of penicillinase will be more involved than if just periplasmic proteins were liberated.

Osmotic shock is reported to release only periplasmic proteins (Heppel, 1967). The process involves first plasmolyzing cells with sucrose in the presence of Tris-HCl and EDTA; this treatment damages the cell envelope. When the damaged cells are exposed to a medium of very low osmotic pressure, such as distilled water, there is a rapid flow of water into the cytoplasm. This expands the inner membrane against the peptidoglycan layer and forces the contents of the periplasm through the damaged cell wall (Heppel, 1967). Although osmotic shock releases 80-100% of periplasmic penicillinase, the procedure requires a minimum of four centrifugations. We have devised a simpler method for extracting penicillinase from E. coli. If the method permits the extraction of periplasmic penicillinase without extracting cytoplasmic proteins, it should be useful for large-scale purification of the enzyme.

Results and Discussion

Our penicillinase assay utilizes paper chromatography to separate benzylpenicillin, a penicillinase substrate, from the hydrolysis product. Since benzylpenicilloic acid has one more carboxyl group than benzylpenicillin (Fig. 1), it should bind more tightly to positively-charged diethylaminoethyl (DEAE) cellulose. In order to test this, *E. coli* K-12 strain WA802/pBR322 was employed. This strain is resistant to high concentrations of ampicillin (e.g., 1 mg/mL) and benzylpenicillin because it harbours plasmid pBR322. When a toluene-permeabilized WA802/pBR322 culture, still in growth medium, was mixed with [^{14}C] benzylpenicillin, some of the substrate was hydrolyzed to [^{14}C] benzylpenicilloic acid. By spotting an aliquot of the reaction mixture on a DEAE-cellulose strip and carrying out ascending chromatography in 0.1 M ammonium acetate, benzylpenicilloic acid (R_f 0.25) could be clearly separated from benzylpenicillin (R_f 0.50). A comparison of radioactivity levels in the two regions of the chromatogram allowed calculation of the amount of benzylpenicillin hydrolyzed, as explained in the 'Experimental Procedures' of this chapter, where the details of the assay are outlined. We found that this assay is not influenced by the presence of 0.1%(w/v) casamino acids, 0.1%(w/v) tryptone, 0.1%(w/v) yeast extract or Tris-buffered growth medium.

At the same time that the specificity and reproducibility of the radioactive penicillinase assay was being established, a simple method of extracting periplasmic penicillinase was developed. To release this enzyme from WA802/pBR322, a number of compounds (e.g., toluene, alcohols and nonionic detergents) were tested; these compounds are known to affect the structure and properties of bacterial membranes, but generally do not denature proteins. It was found that a combination of phenethyl alcohol (PEA) and ethylenediaminetetraacetate (EDTA) released penicillinase, as monitored by the ^{14}C assay described above, although the individual reagents alone did not. The cells were collected by centrifugation, resuspended in mixtures of PEA and EDTA at various concentrations, and shaken for 10 min at room temperature. The mixtures were then centrifuged, and the amounts of penicillinase in the supernatants were compared with those obtained by the osmotic shock method (Neu, 1968). A mixture of 1% (v/v) PEA and 5 mM EDTA (pH 7.0) was found to release the same amount of penicillinase as the osmotic shock method (Table 1). As the same amount of protein was extracted by both methods, the specific activity of the extracted penicillinase was the same. Since osmotic shock is claimed to be specific for periplasmic proteins, (Heppel, 1967), and since PEA-EDTA did not extract more protein than osmotic shock, the

Table 1. Extraction of periplasmic penicillinase from E. coli K12 strain WA802/pBR322.

Assay ^{a)}	PEA-EDTA	Osmotic shock
Penicillinase ^{b)} (units/AU)	0.69	0.67
Protein ^{c)} (mg/AU)	31	33
Specific activity (units/mg)	2.2	2.0

a) Details of the PEA-EDTA and osmotic shock methodologies are given in the 'Experimental Procedures' of this chapter.

b) Penicillinase was measured by our ¹⁴C assay; particulars of the method are given in the 'Experimental Procedures' of this chapter. One unit of penicillinase is defined as the amount of enzyme which hydrolyzes 1 μ mole of benzylpenicillin per minute at pH 7.0 and 30°C.

c) Protein was measured by the method of Bradford (1976).

possibility exists that the PEA-EDTA method is also specific for releasing periplasmic proteins; this possibility is explored further in Chapter 2.

As in the osmotic shock method, some penicillinase (about 5% of total activity) remained associated with the cells even after the PEA-EDTA treatment. However, the cell-associated enzyme hydrolyzed benzylpenicillin in the PEA-EDTA solvent. Therefore, when penicillinase-producing bacteria are resuspended in this solution, penicillinase activity in the suspension approximates total penicillinase activity in the cells. Consequently, the PEA-EDTA method provides a simpler and more reliable penicillinase assay than those involving ultrasonication or osmotic shock treatments. The amounts of benzylpenicilloic acid produced during a 5-min fixed-time assay were directly proportional to enzyme concentration, provided it was below 0.45 units/mL of reaction mixture (Fig 2). If the enzyme concentration in a sample exceeds this range, then the sample must be diluted with PEA-EDTA solvent to make the 5-min assay valid.

The ^{14}C -penicillinase assay was employed to determine the amount of penicillinase accumulating in WA802/pBR322. As seen from Fig 3, the concentration of penicillinase increased in direct proportion to cell density until the end of exponential phase. When the culture entered stationary phase, enzyme production ceased. Similar results were obtained with another penicillinase-producing

Fig 2. Amounts of benzylpenicilloic acid produced during the 5-min fixed-time assay at various enzyme concentrations. Penicillinase was extracted from WA802/pBR322 and assayed as described in 'Experimental Procedures'. The enzyme preparation was variously diluted with the PEA-EDTA solvent. The amounts (in micromoles) of benzylpenicilloic acid produced during the 5-min assay are plotted against concentration of the enzyme (units per millilitre of reaction mixture). The linear region of the plot does not pass through the origin because of non-enzymic degradation of benzylpenicillin, which is constant for a given assay condition.

Fig 3. Accumulation of penicillinase in a culture of WA802/pBR322. The bacteria were grown as described in 'Bacterial Strains and Culture Conditions'. Samples were periodically removed, mixed with toluene to a final concentration of 0.1% (to prevent further protein synthesis), and kept on ice. The cells were harvested by centrifugation and resuspended in the PEA-EDTA solvent. Penicillinase in the suspension was assayed as described in the 'Experimental Procedures' of this chapter. Symbols: ($\square$), units of penicillinase per millilitre of culture; ($\circ$), cell density.

E. coli K-12 strain, 490/pBR322. These results show that the cellular content of penicillinase is constant throughout the exponential growth phase.

The penicillinase assay and extraction methods presented here represent an advancement over previous methods because the assay is specific for the enzyme, and the extraction method is both more reliable than ultrasonication and faster than osmotic shock. The PEA-EDTA method requires only two centrifugations to produce an aqueous extract of penicillinase, while osmotic shock requires from four to six centrifugations, depending on the exact method used (Neu and Heppel, 1965; Heppel, 1967). This translates into the PEA-EDTA method not only being faster than osmotic shock, but also less expensive, particularly if it should find an industrial application such as the commercial extraction of cloned hybrid penicillinase-eukaryotic proteins. Naturally, purification of the hybrid peptide would be simplified if the extraction procedure were specific for periplasmic proteins and did not release cytoplasmic proteins. The specificity of the PEA-EDTA extraction method is examined in Chapter 2.

Experimental Procedures

PEA-EDTA extraction of penicillinase

Bacterial strains WA802/pBR322 and 490/pBR322 were grown in TMM, glucose, phosphate and casamino acids as explained in 'Bacterial Strains and Culture Conditions', to $A_{500} = 3.3$, which corresponds to late exponential phase. Cells were grown in casamino acids so that nutrient availability would not restrict penicillinase production. The cells were collected by centrifugation and resuspended in 1% (v/v) PEA and 5 mM EDTA (pH 7.0), and gently shaken for 10 min at room temperature. Although the mixture could be used directly for the penicillinase assay, the suspension was centrifuged again so that the extraction of penicillinase and total protein could be measured. Since EDTA interferes with the Lowry protein assay (Lowry *et al.*, 1951), protein was assayed by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

Osmotic shock extraction of penicillinase

The details of this method have been previously described (Neu, 1968). Bacterial cells were grown as above, collected by centrifugation and resuspended in 0.9% (w/v) NaCl. The suspension was centrifuged and the pellet resuspended in 0.5 M sucrose in 0.03 M Tris-HCl, pH 8.0 and 1 mM EDTA. After 5 min, the cells were

centrifuged and resuspended in ice-cold water; 10 min later, the suspension was centrifuged again and the supernatant (osmotic shock fluid) was assayed for penicillinase and protein.

¹⁴C-penicillinase assay

[¹⁴C] benzylpenicillin (0.25 μ Ci/mL) and 0.03 M benzylpenicillin solutions in 0.1 M potassium phosphate buffer, pH 7.0, were separately prepared, kept on ice, and used within 30 min. The penicillinase assay mixture consisted of 10 μ L of the [¹⁴C] benzylpenicillin solution, 10 μ L of the benzylpenicillin solution, and 10 μ L of diluted enzyme extract. The reaction mixture with water substituting for extract was used to measure non-enzymic hydrolysis of the substrate. After incubating for 5 min at 30°C, a 10 μ L portion of the mixture was applied to a 2 cm wide strip of DEAE-cellulose (Whatman DE 81). Penicillinase activity stopped immediately upon application. The chromatogram was developed ascendingly in 0.1 M ammonium acetate to 20 cm above the origin (approximately 2 hours) and then dried. Benzylpenicilloic acid was consistently found between 3 and 7 cm and benzylpenicillin between 8 and 14 cm above the origin. The two regions were cut out, placed into scintillation vials, immersed in 1 mL of 1 N HCl for 10 min, and mixed with 10 mL of a detergent-based scintillation fluid (Yeung et al, 1976). The radioactivity counted in a liquid

scintillation counter was used to calculate the amount of benzylpenicillin hydrolyzed, which was then corrected for non-enzymic hydrolysis. One unit of penicillinase is defined as the amount of enzyme which hydrolyzes 1 μ mole of benzylpenicillin per minute at pH 7.0 and 30°C.

CHAPTER TWO

A comparison of protein extraction from Escherichia coli by the PEA-EDTA and osmotic shock methods

Introduction

The similarity in the specific activity of penicillinase extracted by both the PEA-EDTA and osmotic shock methodologies, observed in the previous chapter, suggested that PEA-EDTA treatment of E. coli cells might result in the specific extraction of periplasmic proteins. In order to verify this, PEA-EDTA and osmotic shock extracts were further characterized in order to determine if they differed in any significant manner.

One of the ways in which we tried to establish the specificity of PEA-EDTA extraction was by testing the homogeneity of penicillinase released by both this method and osmotic shock. All protein synthesis takes place within the cytoplasm; therefore, proteins destined for the periplasmic space, like penicillinase, together with proteins meant for export out of the cell, must all first traverse the highly hydrophobic lipid bilayer of the bacterial inner membrane. During the past decade, evidence has mounted that newly-synthesized periplasmic and extra-cellular proteins, still within the cytoplasm and probably bound to the inner membrane (Smith et al.,

1978), differ in structure from more 'mature' protein already situated in the periplasm or extra-cellular environment. The cytoplasmic, or immature forms of most such proteins have been found to have an additional amino acid sequence of 15-30 residues at the amino terminal end of the protein. This sequence, known as the leader or signal sequence, apparently triggers the secretion of the protein across the inner membrane. A number of models have been developed in an effort to explain how secretion occurs (Halegoua and Inouye, 1979; Wickner, 1979; Emr et al., 1980). All of the models suggest that some time after the leader sequence initiates secretion, it is proteolytically cleaved off and the protein assumes its mature conformation.

The amino acid sequences of the signal peptides for several periplasmic proteins have been determined. A striking feature of these sequences is that they all contain a substantial string of hydrophobic amino acids. Furthermore, just prior to the start of the hydrophobic sequence, there is a positively-charged amino acid (either lysine or arginine).

Cytoplasmic, or immature penicillinase has never been isolated from E. coli. However, from the DNA sequence of the penicillinase gene (Sutcliffe, 1978), the amino acid sequence of the signal peptide has been elucidated (Fig 4). As this signal sequence must be cleaved off during the secretion process, it is reasonable to expect

Met - Ser - Ile - Gln - His - Phe - Arg - Val - Ala -
L1   L2   L3   L4   L5   L6   L7   L8   L9

Leu - Ile - Pro - Phe - Phe - Ala - Ala - Phe - Cys -
L10   L11   L12   L13   L14   L15   L16   L17   L18

Leu - Pro - Val - Phe - Ala - His - Pro -
L19   L20   L21   L22   L23   1   2

Fig 4. The amino acid sequence of the penicillinase leader peptide coded on plasmid pBR322. Note the arginine residue at position L7, just before the start of the predominantly hydrophobic region of the sequence (L8-L23). The histidine at position 1 is the amino terminal amino acid of the mature protein. (from Sutcliffe, 1978)

the sequence to be situated on the perimeter of the folded immature protein, readily accessible to whichever proteolytic enzyme is responsible for its removal. Thus, newly synthesized, cytoplasmic (or membrane-bound) penicillinase should differ from the mature, periplasmic form of the enzyme by having a somewhat larger molecular weight, greater hydrophobicity and, due to an additional arginine residue, a slightly higher isoelectric point (pI).

Since immature penicillinase has never been isolated, it is unknown if it exhibits catalytic activity. However, the immature form of another bacterial periplasmic enzyme, alkaline phosphatase, is active (Inouye and Beckwith, 1977). Therefore, the possibility exists that immature penicillinase can be separated from the mature enzyme by virtue of the characteristics listed above, and then identified by its catalytic activity.

In trying to establish if the PEA-EDTA protein extraction method is specific for periplasmic proteins, one of the criteria used involved checking the homogeneity of penicillinase released by both this method and osmotic shock. If heterogeneity were found, then it could indicate that the procedure extracts not only periplasmic penicillinase, but the immature, cytoplasmic form of the enzyme as well. Thus, heterogeneity could suggest that the PEA-EDTA method is not totally specific for periplasmic proteins.

The two methodologies used to detect possible penicillinase heterogeneity relied upon different potential characteristics of the immature enzyme. The first methodology, chromatofocusing, depended upon the extra arginine residue found in the signal sequence to change the pI of the protein. The second methodology, hydrophobic interaction chromatography, relied upon the hydrophobic character of the signal sequence to make immature penicillinase more hydrophobic than the periplasmic form of the enzyme.

Chromatofocusing, a technique developed by Sluyterman et al. (1978 a,b) takes advantage of a protein's behaviour towards a charged surface at pH values above, at and below the protein's pI. When a column is packed with an anion-exchange resin which has buffering capacity over a wide pH range, a pH gradient can be formed within the column by first adjusting the resin to one pH, and then passing through a buffer at a second, lower pH. As the eluting buffer titrates the ionic groups on the resin, a gradient spanning the range from high, initial resin pH to low, eluting buffer pH is formed.

If a protein sample is applied to the column at the start of gradient formation, proteins whose isoelectric points fall somewhere within the gradient will segregate and elute out at their pI. This happens because when the pI of a protein is greater than the pH of its surroundings, the protein carries a positive charge and

migrates down the column in the eluting buffer. However, further down the column the pH of the resin is higher as fewer of the anion-exchange groups have been titrated by the eluting buffer. Thus, as the protein migrates, it encounters resin of ever-increasing basicity. Eventually, when the pH becomes greater than the pI of the protein, the protein reverses its charge and binds to the anion-exchanger. The protein remains bound to the resin until the developing pH gradient causes the pH to drop below the pI of the protein. The protein is then carried along in the eluent buffer again until the pH rises above the pI and it rebinds. This process is repeated until the protein emerges from the column at its isoelectric point. In this chapter, both PEA-EDTA and osmotic shock extracts of WA802/pBR322 were chromatofocused in order to see if any penicillinase heterogeneity could be detected in either or both of these samples. If the column resolved two peaks of penicillinase activity in the PEA-EDTA sample, but only one peak from the osmotic shock extract, we would have to suspect that the PEA-EDTA method was extracting both intracellular and periplasmic enzyme.

The second methodology used to check for possible penicillinase heterogeneity was hydrophobic interaction chromatography. 'Hydrophobic interaction' is something of a misnomer as it is actually the properties of water, and not the properties of hydrophobic groups, that provide the driving force for the interactions.

Strong hydrogen bonds exist between water molecules. In order to dissolve a hydrocarbon in water, a cavity within the water matrix must be formed, and so some of these hydrogen bonds are broken. Those water molecules immediately adjacent to the hydrocarbon cannot form strong hydrogen bonds to the solute; in an effort to compensate for this, they instead form extra hydrogen bonds to neighbouring water molecules. This results in a highly ordered formation of water molecules around the hydrocarbon. When these hydrophobic groups come together into a single, larger cavity, some of the structured water molecules around each solute molecule will be transferred to the bulk water phase. The resulting gain in entropy acts to drive hydrophobic groups together, and so provides the energy for hydrophobic interactions.

The idea of using hydrophobic interactions as a means of fractionating biological molecules is not new, but developments in the field really began in 1970 when Cuatrecasas showed that the use of polymethylene chains as spacer arms in affinity chromatography resulted in enhanced protein adsorption. Soon afterwards, commercial preparations consisting of beaded agarose (a type of polysaccharide) with covalently-linked hydrophobic groups became available. For example, Pharmacia began to market agarose beads whose glucosidic hydroxyl groups were substituted with either phenyl or octyl ligands:

It was initially envisioned that these hydrophobic matrices would be useful in the fractionation of membrane and other lipophilic proteins. However, it was found that hydrophobic interactions between protein and matrix were so intense that it proved almost impossible to elute these proteins from the column. At the same time, papers appeared reporting success in fractionating water-soluble, apparently non-hydrophobic proteins. It soon became evident that most hydrophilic proteins have sufficient hydrophobic amino acid residues on their surfaces to provide interaction sites between protein and matrix. Using PEA-EDTA and osmotic shock extracts of WA802/pBR322, we attempted to detect possible penicillinase heterogeneity by means of a hydrophobic column. Mature penicillinase might bind weakly to the matrix and should easily be eluted, whereas cytoplasmic penicillinase, if it were present, should interact more strongly with the hydrophobic ligands of the matrix and thus be more difficult to elute. The following section describes the evidence we have collected that the PEA-EDTA method is probably as specific for the extraction of periplasmic proteins from E. coli as osmotic shock is.

Results and Discussion

The specificity of the PEA-EDTA extraction method was tested in the following ways: 1) polyacrylamide gel electrophoresis of both PEA-EDTA and osmotic shock extracts of E. coli; 2) assays on both types of extracts for the presence of the cytoplasmic enzymes β -galactosidase and cyclic adenosine monophosphate (cAMP) phosphodiesterase; 3) chromatofocusing of penicillinase from PEA-EDTA and osmotic shock extracts of WA802/pBR322 and 4) attempted fractionation of penicillinase from these two samples on hydrophobic columns.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS) separates proteins on the basis of molecular weight. SDS, a negatively-charged detergent, binds to proteins and denatures them. The elongated peptides can then be forced to migrate through a small-pore gel by the application of an electric current. The rate at which a protein can work its way through the gel is inversely related to its size: the smaller the peptide, the faster it migrates. By staining the gel with a protein dye, a panoramic representation of the proteins in the sample is obtained. SDS-polyacrylamide gel electrophoresis was used in order to see if there were any significant differences in the proteins extracted by the PEA-EDTA and osmotic shock methods. As seen from Fig 5, protein patterns of samples prepared by both these methods

Fig 5. SDS-polyacrylamide gel electrophoresis of PEA-EDTA and osmotic shock extracts of WA802 and WA802/pBR322. Lanes A-D contain 10 μ L of the following: Lane A, PEA-EDTA extract of WA802/pBR322; Lane B, osmotic shock extract of WA802/pBR322; Lane C, PEA-EDTA extract of WA802 and Lane D, osmotic shock extract of WA802. Lane E contains β -lactoglobulin, which has a molecular weight of 1.85×10^4 daltons. The arrow points to protein bands in Lanes A and B which are missing from Lanes C and D. Judging from the position of these bands on the gel, and knowing that the molecular weight of penicillinase is 2.89×10^4 (Ambler and Scott, 1978), it is probable that these bands are penicillinase.

are very similar. Furthermore, judging from the intensity of the bands, the degree of protein extraction by the two methods is comparable. We believe these results are strong evidence that the PEA-EDTA extraction method is not only as specific for periplasmic proteins as osmotic shock is, but that it is also as efficient.

The specificity of the PEA-EDTA method was further tested by checking for the presence of intracellular enzymes. If both the PEA-EDTA and osmotic shock methods are entirely specific for the extraction of periplasmic proteins, then ideally there should be no indication of cytoplasmic enzyme activity in either of the two samples.

In order to test for cAMP phosphodiesterase activity, PEA-EDTA and osmotic shock extracts of WA802/pBR322 in late exponential phase were prepared. Using the assay method described in the 'Experimental Procedures' of this chapter, I was unable to detect any sign of cAMP phosphodiesterase activity in either of the two extracts.

To test for possible β -galactosidase extraction, E. coli strain 3300, which is constitutive for the production of this enzyme, was used. A minute amount of enzyme activity could be detected in both 3300 extracts: the PEA-EDTA sample contained 6.2 units β -galactosidase per mL extract, and the osmotic shock sample 7.1 units/mL. Why this β -galactosidase activity was extracted is unknown; it may be related to the large quantity of the enzyme found in 3300 (Pardee et al., 1959). Whatever the

origin of the β -galactosidase activity, it is important to note that no more was extracted by the PEA-EDTA method than was extracted by osmotic shock.

The third method of testing the specificity of PEA-EDTA extraction involved chromatofocusing extracts of WA802/pBR322 and checking for the possible presence of immature, or cytoplasmic, penicillinase. The protein product of the penicillinase gene carried on plasmid pBR322 has a pI of 5.4 (Matthew and Hedges, 1976). Because of the arginine residue on the signal sequence, immature penicillinase would be expected to have a slightly higher pI. To resolve possible cytoplasmic penicillinase from the periplasmic form of the enzyme, a shallow pH gradient of 6-5 and a tall, narrow column of anion-exchange resin were used. According to the Pharmacia Product Guide manual, these conditions should result in maximum resolution of proteins with similar isoelectric points in the pH range 6-5.

PEA-EDTA and osmotic shock extracts of WA802/pBR322 were chromatofocused in an identical manner. All of the one hundred fractions off each column were rapidly checked for penicillinase activity in order to identify the position of the penicillinase peaks. In each chromatofocusing run, a minute, barely discernible amount of penicillinase activity was found in all the fractions; however, as Table 2 indicates, most penicillinase activity was efficiently focused into just a few fractions. In

Table 2. The specific activity of the penicillinase peak fractions of chromatofocused PEA-EDTA and osmotic shock WA802/pBR322 extracts:

Sample	Fraction number	Penicillinase ^{a)} (units/mL)	Protein ^{b)} (mg/mL)	Specific activity (units/mg)
PEA-EDTA ^{c)}	43	0.34	2.1	0.16
	44	16	2.1	7.6
	45	110	2.2	50
	46	149	2.2	68
	47	125	2.2	57
	48	23	2.1	11
	49	2.0	2.1	0.95
	50	0.46	2.1	0.22
osmotic ^{d)} shock	41	0.16	2.0	0.08
	42	0.97	2.0	0.49
	43	9.2	2.1	4.4
	44	98	2.2	44
	45	179	2.2	81
	46	114	2.0	57
	47	36	2.0	18
	48	1.8	2.2	0.82
	49	0.47	2.0	0.23

a) Penicillinase activity was measured by the microiodometric method described in the 'Experimental Procedures' of this chapter.

b) Protein was measured by the method of Lowry et al. (1951)

c) pH of combined fractions 43-50 was 5.45

d) pH of combined fractions 41-49 was 5.40

both the PEA-EDTA and osmotic shock extracts, only one peak of penicillinase activity was found. Protein assays on each peak fraction allowed the calculation of the specific activity of penicillinase extracted by the two methods. It is seen from Table 2 that approximately the same amount of enzyme was extracted in both cases.

The apparent homogeneity of penicillinase may indicate that the cytoplasmic form of the enzyme is not extracted by either of the two methods. On the other hand, immature penicillinase may have no activity, and so cannot be detected. Alternatively, the pI of immature enzyme may be the same as that of periplasmic penicillinase. The effect of the positive charge of the signal sequence's arginine residue would be negated if the amino-terminal methionine residue is formylated. If this is the case, then chromatofocusing would be unable to resolve mature and immature penicillinase.

In a further attempt to detect the possible extraction of cytoplasmic penicillinase, PEA-EDTA extract of WA802/pBR322 was applied to an octyl-agarose column. Two methods of preferentially eluting the more hydrophilic mature enzyme from any cytoplasmic penicillinase that might be present in the sample were tried. Protein elution from hydrophobic matrices can often be accomplished by adding a component which interacts so strongly with the hydrophobic ligands of the matrix that absorbed substances are displaced from the column. Common

displacers are detergents, such as sodium deoxycholate, and polarity reducing agents such as ethylene glycol (Hjerten, 1973). Both these compounds were tried in an effort to fractionate penicillinase bound to octyl-agarose.

It was found that penicillinase could be eluted as a broad peak by washing the column with 0.1% sodium deoxycholate. However, not all activity could be displaced in this manner. Even after exhaustive washing with the detergent, a small amount of penicillinase activity continued to be eluted from the column; furthermore, substantial activity remained bound to the matrix. Similar results were obtained using osmotic shock extract of WA802/pBR322. In an attempt to increase resolution, a linear gradient of 0.1-0.5% sodium deoxycholate was pumped through the column. However, the detergent solution soon became so viscous that it was unable to flow through the octyl-agarose bed. Therefore, this elution method was abandoned in favour of using ethylene glycol. However, the elution profile obtained using the polarity reducing agent was similar to that obtained with sodium deoxycholate. When PEA-EDTA extract was bound to octyl-agarose, penicillinase activity was displaced by 40% ethylene glycol in a very broad peak. Elution with a stepwise gradient of the diol resulted in a new broad peak being eluted each time the ethylene glycol concentration was raised. It became apparent that

penicillinase fractionation on an octyl-agarose column would not answer the question of whether or not the PEA-EDTA method extracts cytoplasmic penicillinase. Nevertheless, judging from the other data presented in this chapter, it is probable that the fast and simple PEA-EDTA method is not only as specific for periplasmic proteins as osmotic shock is, but also as efficient.

An interesting observation from the above studies with octyl-agarose is that penicillinase binds strongly to a hydrophobic matrix, and is apparently very active when so immobilized. When a coloured penicillinase substrate solution was passed through an octyl-agarose bed which had been previously exposed to either PEA-EDTA or osmotic shock extract of WA802/pBR322, the substrate was immediately decolorized, indicating that a high level of penicillinase activity was bound to the column. Even after washing such a column with 100% ethylene glycol, substantial penicillinase activity remained bound to the matrix. This observation suggested that hydrophobic interactions might be an effective means of producing active immobilized enzyme preparations; this possibility is further examined in the following chapter.

Experimental Procedures

SDS-polyacrylamide gel electrophoresis

The system used for preparing and running SDS pH-discontinuous gels was that of Lugtenberg et al. (1975). This system involves the use of two gels, cast one on top of the other. The pH and acrylamide pore sizes of the gels are chosen so that the top gel serves to stack the protein sample into a very thin zone, and the bottom, or running gel, separates the proteins on the basis of size. Such discontinuous systems give better resolution of protein bands than single-gel systems.

Samples for electrophoresis were prepared by growing bacterial strains WA802 and WA802/pBR322 each in 2 X 100 mL of TMM supplemented with glucose, phosphate and methionine, as described in 'Bacterial Strains and Culture Conditions'. All four cultures were grown to $A_{500} = 1.0$, which corresponds to mid-log phase. One culture of both WA802 and WA802/pBR322 was osmotically shocked in 2.0 mL ice-cold water, as explained in the 'Experimental Procedures' of Chapter 1. The second set of cultures was treated with 2.0 mL 1% PEA in 5 mM EDTA, pH 7.0, as previously described. The PEA-EDTA extracts were dialysed against three changes of ice-cold water for a total of six hours to remove PEA and EDTA which might otherwise have interfered with the electrophoresis. All four cell extracts were then freeze-dried and resuspended in 0.5 mL

protein resuspension buffer (Lugtenberg et al., 1975). After boiling for 5 min, 10 μ L of each sample were loaded into wells formed in the stacking gel. The stacking gel was about 35 mm high, and the running gel about 115 mm high. Overall dimensions of the gel were 80 X 140 X 2.7 mm. Once samples were loaded, electric current was applied so that the negatively-charged, SDS-coated proteins were forced to move through the gel in the direction of the anode. At 50 mA/gel, the tracking dye in the protein resuspension buffer traversed the 3%(w/v) acrylamide stacking gel in 1.5 hours, and the 11%(w/v) acrylamide running gel in about 3.5 hours. The temperature of the electrophoresis unit was maintained at 15°C. At the completion of the run, protein bands were fixed to the running gel by soaking overnight in a solution of 4%(w/v) sulphosalicylic acid and 12.5%(w/v) trichloroacetic acid. Gels were then stained for two hours in a solution of 27%(v/v) ethanol, 10%(v/v) acetic acid, 0.5%(w/v) cupric sulphate and 0.04%(w/v) Coomassie brilliant blue. Protein bands were visualized by destaining the gel for several days in 12%(v/v) ethanol and 7%(v/v) acetic acid.

β -galactosidase enzyme assay

E. coli strain 3300 was grown in TMM supplemented with glucose and phosphate to $A_{500} = 2.0$, which corresponds to late exponential phase. One mL of culture was osmotically shocked in an equal volume of ice-cold water, and another mL of culture treated with an equal volume of PEA-EDTA; both extraction methods have been previously described in the 'Experimental Procedures' of Chapter 1.

β -galactosidase activity in the two extracts could be conveniently assayed by using o-nitrophenol- β -D-galactopyranoside (ONPG) as substrate. The enzyme cleaves ONPG and releases o-nitrophenol which absorbs strongly at 420 nm at alkaline pH. As the intensity of absorption is proportional to the amount of substrate hydrolyzed, the amount of enzyme activity in a sample can be easily determined. It should be noted that ONPG is an artificial chromogenic substrate; the natural substrate of β -galactosidase is lactose, which the enzyme hydrolyzes to galactose and glucose.

To 2.0 mL ONPG (0.8 mg/mL in 0.05 M sodium phosphate buffer, pH 7.0), 1.0 mL of enzyme extract was added. After vortexing, the enzyme reaction was allowed to proceed for 30 min at 30°C; the reaction was halted by the addition of 1.0 mL 1.0 M Na_2CO_3 . The optical density of the reaction mixtures was measured at 420 nm against blanks of 2.0 mL ONPG, 1.0 mL of the Na_2CO_3 solution, and

1.0 mL of either water or PEA-EDTA solution. One unit of β -galactosidase activity is defined as that amount of enzyme which hydrolyzes 1 μ mole of ONPG per hour at 30°C in the presence of 2 mM substrate in 0.05 M sodium phosphate, pH 7.0. The extinction co-efficient of o-nitrophenol is assumed to be 4.7×10^3 litres/mole/centimetre.

cAMP phosphodiesterase enzyme assay

The presence of cAMP phosphodiesterase activity in PEA-EDTA and osmotic shock extracts of *E. coli* was tested by measuring the rate of degradation of [^{32} P]-labelled cAMP to 5'-AMP by the extracts. [^{32} P] cAMP was prepared and assayed for purity by the method of Yamazaki *et al.* (1979). Osmotic shock and PEA-EDTA extracts of WA802/pBR322 in late exponential phase were prepared as previously described. cAMP phosphodiesterase in the extracts was measured by mixing 10 μ L extract, (or water in the case of the control), 10 μ L [^{32} P] cAMP and 10 μ L 45 mM cAMP and incubating at 37°C. At 0, 15, 30 and 60 min after the start of the reaction, 5 μ L aliquots of the reaction mixture were removed and spotted on alumina plates to which 5'-AMP and cAMP markers had already been applied. The plate was then developed overnight in the ascending direction in ethanol and 1 M ammonium acetate (13:7 v/v). After air drying, 5'-AMP and cAMP markers were located by UV visualization at 254 nm.

Autoradiography of the plate allowed qualitative determination of cAMP phosphodiesterase activity. Quantitative results could be obtained by cutting out pairs of cAMP and 5'-AMP spots and determining the ratio of radioactivity between them by means of scintillation counting.

Microiodometric determination of penicillinase activity

The iodometric penicillinase assay of Novick (1962), rather than the ^{14}C -penicillinase assay introduced in the previous chapter, was used in several experiments presented above because it allowed the rapid visual detection of peaks of penicillinase activity in a large number of column fractions. In addition, the protein samples applied to the various columns were probably free of iodine-absorbing compounds. The reagents used were: 0.2% (w/v) soluble starch in 0.1 M phosphate buffer, pH 7.0; 0.08 M iodine reagent in 3.2 M potassium iodide (0.2 g of I_2 plus 5.33 g of KI in 10 mL water) and 0.20 mM benzylpenicillin in 0.1 M phosphate buffer, pH 7.0. Starch-iodine solution was prepared by adding 0.15 mL of iodine reagent to 100 mL of starch solution; this gave a final iodine concentration of 0.12 mM. To determine the penicillinase activity of a column fraction, the rate of decolorization of the starch-iodine complex by penicilloic acid at 620 nm was followed. This was accomplished by mixing in a cuvette 1.0 mL starch-iodine solution, 1.0 mL

benzylpenicillin solution and 0.9 mL 0.1 M phosphate buffer, pH 7.0; the reaction was started by adding 0.1 mL enzyme (column fraction). The enzyme was suitably diluted so that total decolorization, as determined against a phosphate buffer blank, took about 20 min; penicillinase activities higher than this tended to result in an underestimation of the enzyme. By measuring absorbance of the cuvette at various times after the reaction became linear, the rate of benzylpenicillin hydrolysis by penicillinase could be determined. The amount of iodine used in the assay corresponds to 30 nmoles of penicillin. At this concentration, the optical density at 620 nm decreases from 1.20 to 0 when the penicillin is completely hydrolyzed by the enzyme. One unit of penicillinase activity is defined as the amount of enzyme which hydrolyzes 1 μ mole of substrate per min at 30°C, pH 7.0.

Very rapid identification of penicillinase peak fractions was accomplished by adding an aliquot of the fraction to about half a millilitre of the starch-iodine-penicillin solution. Those column fractions which caused complete decolorization to occur within 5-10 min were assayed more closely for penicillinase activity by the method detailed above.

Chromatofocusing of penicillinase

Since maximum resolution of proteins with pI values near 5.4 was required, a shallow gradient of pH 6-5 was used. The anion exchange resin, called Polybuffer exchanger (PBE) 94, was equilibrated in a sintered glass filter with degassed 0.025 M histidine-HCl, pH 6.2. Once pH equilibration was achieved, the exchanger was packed into a 50 X 0.7 cm (i.d.) column to a bed height of 40 cm by half-filling the column with histidine buffer, pouring in a slurry of PBE 94, and allowing a settled bed to form under gravity. The bed was topped by a 5 cm high layer of Sephadex G-25 coarse; the Sephadex layer permitted gradual and even sample application to the PBE 94 bed.

Two 250 mL cultures of WA802/pBR322 were grown in TMM supplemented with glucose, phosphate and methionine to $A_{500}=1.0$ (mid-log phase). Proteins were extracted into 4.0 mL of either ice-cold water or PEA-EDTA by the methods previously described. The PEA-EDTA extract was dialysed against three changes of ice-cold water for a total of six hours to remove PEA and EDTA which might otherwise have interfered with chromatofocusing. Extracts were divided into 1 mL aliquots and stored frozen at -90°C .

One mL of extract was applied to the chromatofocusing column which had been pre-equilibrated to 4°C . Once the sample had run into the Sephadex G-25 layer, protein elution from the column was initiated by passing a 1:10

dilution of degassed Polybuffer 74-HCl, pH 5.0, through the column. Passage of Polybuffer through the bed was achieved by gravity. With 100, 20 drop fractions collected, the entire run, which was carried out at 4°C, took about 15 hours. At the completion of the run, the chromatofocusing column was washed with three bed volumes of 1 M NaCl to remove bound substances, and then was re-equilibrated to the starting pH by passing through ten bed volumes of degassed 0.025 M histidine-HCl, pH 6.2 buffer. The column was then ready to chromatofocus another sample.

Fractions off the column were rapidly checked for penicillinase activity by mixing 20 μ L of fraction with 0.5 mL of the starch-iodine-penicillin solution described above. Once the peak fractions were identified in this manner, comprehensive penicillinase assays were carried out using the iodine method previously described. Protein assays by the method of Lowry et al. (1951) on the peak fractions allowed calculation of the specific activity of penicillinase focused by the column.

Hydrophobic interaction chromatography of penicillinase

Sample extracts prepared for chromatofocusing were also used for hydrophobic fractionation studies. Octyl-agarose columns were prepared by half-filling a 10 x 0.5 cm (i.d.) column with 0.01 M phosphate buffer, pH 7.0 and pouring in enough slurry of octyl-agarose to

give a final settled bed height of 2 cm. The matrix was then equilibrated with the phosphate buffer, and the bed covered with two disks of filter paper to ensure that it would not be disturbed by subsequent procedures. A fresh bed of octyl-agarose was laid for each experiment.

A 0.1 mL aliquot of either osmotic shock or PEA-EDTA extract was run into the matrix bed and allowed to bind for 30 min. Any unbound protein was eluted by washing the column with several bed volumes of phosphate buffer. Penicillinase fractionation was attempted by pumping a 40 mL linear gradient of 0.1-0.5% (w/v) sodium deoxycholate in phosphate buffer through the bed and collecting 15 drop fractions; the fractions were rapidly monitored for penicillinase activity as described above.

In the case of enzyme elution with ethylene glycol, a stepwise gradient of 20, 40, 60, 80 and 100% (v/v) ethylene glycol was used. The twelve, 15 drop fractions collected for each step were checked for penicillinase activity as described above. To see if any activity remained bound to the column even after washing with 100% ethylene glycol, starch-iodine-penicillin solution was passed through the column; decolorization of the solution indicated that active penicillinase was still bound to the column.

CHAPTER THREE

Enzyme immobilization through hydrophobic interactions

Introduction

The observation, described in Chapter 2, that penicillinase can be completely adsorbed to octyl-agarose and still retain enzymic activity, suggested that hydrophobic interactions might be an effective means of immobilizing enzymes. Previous studies into the feasibility of employing hydrophobic interactions for enzyme immobilization centred on the enzyme β -amylase. Caldwell *et al.* (1976) found that β -amylase adsorbed to agarose gels substituted with six-carbon chain ligands:

The bound β -amylase preparation, maintained at 25°C, was washed continuously with a 1%(w/v) starch solution. Although initially large amounts of enzyme were eluted from the matrix, by the tenth day elution had decreased to negligible values. Largely due to this loss, amylase activity declined by 50% in the first ten days of column operation; during the next forty days, activity decreased by a further 40%, so that after fifty days of operation, only 10% of initial β -amylase activity remained. The

stability of this immobilized enzyme system was found to be temperature sensitive. After ten days at 35°C, only one-tenth the activity remained compared to an identical preparation maintained at 25°C. Temperature sensitivity could be due to an increased rate of protein thermal denaturation at elevated temperatures. It is unlikely to be due to a weakening of hydrophobic interactions which, according to Scheraga *et al.* (1962), increase with increasing temperatures up to 60°C.

If enzymes can be efficiently immobilized through hydrophobic interactions, then this would be a very simple and gentle means of producing immobilized enzyme preparations. As enzymes would not be exposed to the harsh conditions commonly encountered with co-valent binding techniques, denaturation could be minimized. Furthermore, there should be relatively free diffusion of substrate and product to and from an enzyme adsorbed on the surface of a hydrophobic matrix.

In order to investigate the applicability of hydrophobic interactions to the immobilization of enzymes, five enzymes of potential or proven commercial value were bound to hydrophobic matrices, and the behaviour of these preparations was studied. The five enzymes were invertase, alkaline phosphatase, β -galactosidase, β -glucosidase and histidase. Invertase is an important enzyme to the food industry, as it hydrolyzes sucrose into glucose and fructose. It is widely used in the production

of high fructose-glucose syrup. Alkaline phosphatase removes orthophosphate from orthophosphoric monoesters; the enzyme has been used to remove 5' terminal phosphates from plasmid DNA cut by restriction enzymes. Once these phosphates are removed, the plasmid DNA cannot self-ligate, and circle formation can occur only after the insertion of a DNA fragment containing 5'-phosphorylated termini. Using this method, the DNA fragment can be efficiently cloned (Ullrich et al., 1977). The third enzyme used, β -galactosidase, hydrolyzes lactose into galactose and glucose. Lactose is the principle sugar found in milk and milk products. Because lactose cannot be utilized by many Asian and African adults, it must be removed in order to make milk products suitable for consumption by these people. Thus, β -galactosidase could be used to convert lactose into galactose and glucose in dairy products destined for overseas markets. β -glucosidase hydrolyzes cellobiose into two glucose units. As cellobiose is the final product of cellulose degradation by exo- and endo-cellulases, β -glucosidase may play an important role in the conversion of biomass to ethanol, as yeast will only ferment glucose, and not cellobiose, to ethanol. Finally, histidase catalyzes the conversion of histidine into urocanic acid, a compound which exhibits strong UV absorbing properties. This makes urocanic acid a valuable and common ingredient in sun-screen preparations.

In the β -amylase study cited above, no enzyme activity was found in a ten day old preparation of β -amylase, immobilized on agarose substituted with three-carbon chain ligands. Evidently, the increased hydrophobicity of hexyl-agarose imparted stability to the matrix-enzyme system. However, excessively long hydrophobic ligands may cause non-specific denaturation of proteins (Rosengren et al., 1975); therefore, we chose to do our studies using commercially available agarose substituted with aliphatic dodecyl groups:

dodecyl-agarose

It was hoped that dodecyl ligands would provide strong hydrophobic attachment between enzyme and matrix without denaturing the protein. As dodecyl-agarose contains an isourea linkage between the agarose backbone and the hydrophobic ligand, a positive charge is introduced into the system. It should therefore be noted that ionic interactions may have augmented hydrophobic binding in the following experiments. Furthermore, as substrate flow rate through the various column beds was not controlled, the following results should be regarded as being qualitative in nature.

Results and Discussion

All five enzymes, when percolated through dodecyl-agarose columns, were immobilized by the hydrophobic matrix in an active form, although the performance of the various columns with time varied from one enzyme to another. Considering first the example of invertase, it is seen from Table 3 that a preparation maintained at 30°C and assayed with 0.1 M sucrose as substrate exhibited no detectable decrease in activity over a four month period. It was postulated that low substrate concentration was underestimating the performance of the column, thus masking the effects of enzyme denaturation. Therefore, a similar column was assayed using 1.0 M sucrose as substrate; column performance remained unchanged over a three week assay period.

The effect of elevated temperatures on dodecyl-agarose bound invertase was studied by maintaining a preparation at 45°C and assaying with 1.0 M sucrose. As seen from Table 3, such a column had an apparent half-life of ten days. These results, similar to those obtained by Caldwell et al. (1976) using β -amylase, indicate that at least some hydrophobically-immobilized enzyme preparations are less stable at elevated temperatures. Temperature sensitivity may be due to an increase in hydrophobic interactions with increasing temperatures up to 60°C. As

Table 3. The performance of dodecylagarose-bound invertase columns, maintained at constant temperature and assayed with either 0.1 M or 1.0 M sucrose. Activity is expressed in terms of mole reducing sugar per litre column eluent.^{a)}

Time after immobilization (weeks)	30°C, 0.1 M	30°C, 1.0 M	45°C, 1.0 M
0	0.17	0.73	0.60
1	0.17	0.73	0.36
2	0.16	0.76	0.24
3	0.17	0.73	0.17
4	0.16		0.14
8	0.16		
12	0.17		
16	0.16		

a) Columns were assayed three times a week, in duplicate. Thus, values given in this Table are an average of several activity determinations.

enzymes are not bound to the matrix through rigid covalent bonds, the proteins retain considerable freedom to change conformation as they experience increased thermal motion. Conformational changes would undoubtedly result in the exposure of new hydrophobic amino acid binding sites. Interactions between the ligands on the matrix and these newly-exposed residues may result in partial protein unfolding, which in turn could lead to enzyme denaturation and loss of activity.

The behaviour of the four other dodecyl-agarose bound enzymes studied during the course of this investigation is summarized in Table 4. Overall, the performance of these preparations was good, although immobilized L-histidase did appear to lose activity quite rapidly. Nevertheless, the results of this study provide qualitative evidence that hydrophobic interactions are an effective and gentle means of immobilizing enzymes. Mild adsorption conditions would be expected to help minimize enzyme denaturation resulting from immobilization. This study also suggests that nonpolar amino acids are common on the surfaces of many, and perhaps most, water-soluble proteins, and that these residues are available for interactions with hydrophobic ligands. Thus, hydrophobic interactions may provide an almost universal means of immobilizing enzymes, with one type of hydrophobic matrix, such as dodecyl-agarose, being suitable for a wide variety of proteins.

Table 4. The performance of dodecylagarose-bound enzyme preparations maintained at 30°C. Activity is expressed in terms of product concentration in column eluent (mole/litre).^{a)}

Time after immobilization (weeks)	Alkaline phosphatase ^{b)}	β -glucosidase ^{c)}	β -galactosidase ^{d)}	L-histidase ^{e)}
0	5.5×10^{-5}	2.5×10^{-4}	9.6×10^{-4}	4.7×10^{-5}
1	5.1×10^{-5}	2.5×10^{-4}	9.6×10^{-4}	3.4×10^{-5}
2	4.8×10^{-5}	2.5×10^{-4}	9.6×10^{-4}	1.8×10^{-5}
3	4.3×10^{-5}	2.5×10^{-4}	9.6×10^{-4}	9.6×10^{-6}
4	3.8×10^{-5}	2.5×10^{-4}		
6	3.2×10^{-5}			
8	2.8×10^{-5}			
10	2.1×10^{-5}			
12	2.0×10^{-5}			
14	2.0×10^{-5}			
16	2.0×10^{-5}			

a) As columns were assayed in duplicate several times a week, the above are averaged activity values.

b) Substrate was 1 mM p-nitrophenyl phosphate

c) Substrate was 1 mM p-nitrophenyl- β -D-glucoside

d) Substrate was 2 mM o-nitrophenyl- β -D-galactoside

e) Substrate was 0.03 M L-histidine

A possible merit to the use of hydrophobically-immobilized enzymes in industry is that it should be possible to periodically recycle the hydrophobic matrix of an enzyme reactor when column activity decreases to an unacceptable level; this could be done by eluting old enzyme and readsorbing a freshly-active preparation. The importance of matrix reusability may be better appreciated if one considers that an industrial reactor may contain several thousand litres of immobilized enzyme. Increased public awareness with regard to pollution problems has elevated used matrix disposal costs and has made regeneration or recycling a critical issue (Kent et al., 1978). A further possible advantage of hydrophobic interaction immobilization is the feasibility of having multienzyme systems within a single reactor.

Two limitations to the use of hydrophobic interactions for enzyme immobilization can be foreseen. The first is a hypothetical limitation, and concerns the possibility of enzyme elution from the matrix. When substrate comes in contact with an enzyme's active site, the enzyme undergoes conformational changes; such changes would likely result in a redistribution of surface hydrophobic amino acids, and so of protein-matrix binding sites. Therefore, strongly hydrophobic residues on the surface of the protein might be replaced by more hydrophilic residues; the resulting decreased number of hydrophobic interactions could cause protein elution. A

1-

second limitation is that only enzymes acting upon hydrophilic substrates, and producing hydrophilic products, could be immobilized to hydrophobic matrices.

Although the use of dodecyl-agarose is acceptable for laboratory-scale enzyme immobilization, it would be prohibitively expensive to use agarose as a matrix in a commercial reactor. Thus, industrial reactors containing hydrophobically-bound enzymes could be feasible only if the cost and performance of such a system were competitive with other commercial immobilized enzyme preparations. The question of how to produce an inexpensive hydrophobic matrix is addressed in the next chapter.

Experimental Procedures

Column preparation and assay

Jacketed glass columns (20 cm X 1 cm, i.d.) were connected in series to a thermostated circulating water bath maintained at constant temperature. Dodecyl-agarose beds were laid by half filling a column with water, and pouring in enough slurry of matrix to make a final settled bed height of 5 cm. The bed was covered with two disks of filter paper to ensure that the matrix bed would not be disturbed by subsequent procedures.

Ten mg of enzyme was dissolved in 1.0 mL of appropriate buffer; this was slowly percolated through the column bed and the enzyme was left to bind overnight. The various enzymes were assayed as follows:

a) Invertase

As invertase hydrolyzes sucrose into glucose and fructose, invertase activity can be assayed by observing the difference in reducing power between the column eluent and the column feed solution. The reducing power is measured using 3,5-dinitrosalicylic acid (DNS). DNS is reduced by glucose and fructose to 3-amino-5-nitrosalicylic acid, which absorbs strongly at 540 nm. DNS reagent was prepared by dissolving 5.0 g DNS, 8.0 g NaOH and 150 g sodium potassium tartrate in sufficient water so that the final volume of the solution was 500 mL.

The performance of invertase columns was monitored by passing several bed volumes of either 0.1 M or 1.0 M sucrose through the bed. As this was achieved by gravity, flow rate through these and other columns to be subsequently described was not controlled. The fifth and sixth bed volumes of column eluent were assayed for reducing sugar. Prior to each assay, a blank solution and series of standards were prepared as follows, where values are in mL:

Substance	Blank	Std.1	Std.2	Std.3
5 mM glucose + 5 mM fructose	-	0.4	0.8	1.2
0.3 M or 3.0 M sucrose	1.0	1.0	1.0	1.0
water	2.0	1.6	1.2	0.8

To the above blank and standard solutions, 2.0 mL of DNS reagent was added. DNS was likewise added to 3.0 mL of eluent, which had been diluted as necessary to keep spectrometer readings within the realm of the standard solutions. After vortexing, colour in the tubes was developed by placing them in boiling water for 10 min, cooling, diluting to 20 mL with water, and determining the optical density of each tube at 540 nm against the blank. As the amount of reducing sugar in the third standard is 4 μ mol/mL, the amount of sucrose hydrolyzed by immobilized invertase could be determined.

After an assay determination, residual sugars in the matrix bed were removed by washing the column with water.

b) Alkaline phosphatase

Alkaline phosphatase removes orthophosphate from orthophosphoric monoesters. It can be conveniently assayed through the use of the chromogenic substrate, p-nitrophenol phosphate. The p-nitrophenol moiety released as a result of enzymic action exhibits yellow colour at alkaline pH, with the intensity of colour being proportional to the amount of substrate hydrolyzed. The extinction co-efficient of p-nitrophenol is assumed to be 1.62×10^4 litres/mole/centimetre.

Several bed volumes of 1 mM p-nitrophenol phosphate in 0.1 M Tris-HCl, pH 8.0, were passed through the column. The fifth and sixth bed volumes of column eluent were collected, and their absorbance at 420 nm was measured against a blank containing 3.0 mL of the substrate. Between assays, the alkaline phosphatase column bed was stored under water.

c) β -glucosidase

β -glucosidase hydrolyzes cellobiose to yield two glucose units, and is conveniently assayed with the chromogenic substrate p-nitrophenol- β -D-glucoside. As with the alkaline phosphatase assay, p-nitrophenol is released and its absorbance at alkaline pH can be measured at 420 nm.

Several bed volumes of 1 mM p-nitrophenol- β -D-

glucoside were passed through the column; 2.0 mL of the fifth and sixth bed volumes of eluent were collected and each was made alkaline by the addition of 1.0 mL 1 M Tris base. Optical density of the product was determined against a blank of 2.0 mL substrate and 1.0 mL of the Tris solution. The column bed was stored under water.

d) β -galactosidase

β -galactosidase catalyzes the hydrolysis of lactose into galactose and glucose. The enzyme is easily assayed using o-nitrophenol- β -D-galactoside as substrate since the o-nitrophenol moiety absorbs strongly at 420 nm at alkaline pH. The extinction co-efficient of o-nitrophenol is assumed to be 4.7×10^3 litres/mole/centimetre.

The substrate at a concentration of 2 mM was passed through the column; 1.0 mL 1 M Na_2CO_3 was added to 3.0 mL final eluent. The intensity of the yellow colour was measured against a blank of 3.0 mL substrate and 1.0 mL 1 M Na_2CO_3 . The column bed was stored under water.

e) L-Histidase

L-Histidase catalyzes the conversion of L-histidine to urocanic acid, a compound which exhibits strong UV absorbing properties. L-histidine at a concentration of 0.03 M in 3 mM MgCl_2 and 3 mM dithiothreitol was passed through the column. The optical density of the fifth and sixth column volumes of eluent was determined at 277 nm

against a substrate blank. The column was stored in the MgCl_2 -dithiothreitol solution. The extinction co-efficient of urocanic acid is assumed to be 1.88×10^4 litres/mole/centimetre.

CHAPTER FOUR

The synthesis of hydrophobic derivatives of cellulose

Introduction

The results presented in the previous chapter suggest that agarose substituted with hydrophobic ligands can be used to immobilize enzymes. However, there are several disadvantages to the use of agarose as a matrix in an industrial reactor. The first is cost. Agarose is one of two groups of polysaccharides which comprise agar. The purification procedures necessary to isolate agarose from its seaweed source contribute to making agarose an expensive material for use on a large scale. Furthermore, agarose is unstable at temperatures above 40°C, and its gel structure may give rise to diffusional problems, as the movement of substrate and product to and from enzymes immobilized within the gel matrix may be restricted. In addition, gels in general are deformable and tend to compact, giving rise to large pressure drops across the reactor bed and poor substrate flow rates.

Cellulose has a number of characteristics which make it a suitable matrix for use in enzyme reactors. Cellulose is inexpensive, and when packed into a column, its structural rigidity minimizes compression. Therefore, good substrate flow rates are maintained and reactor

conditions may be optimized. Cellulose is quite resistant to microbial attack and because of its heat stability, is easily sterilized; this quality may be particularly important if the reactor is producing a product for either the food or pharmaceutical industries. Furthermore, since cellulose fibres are relatively non-porous, enzyme immobilization will occur on the external surface of the fibre. As the enzymes are thus in immediate contact with the substrate solution, diffusional effects are minimized.

Despite these advantages, the chemical makeup of cellulose may impose limitations on the degree to which hydrophobic ligands might be introduced into its structure. The linear chains of 1,4-linked β -D-glucose residues of which cellulose is comprised are organized in fibres of a high degree of crystallinity. This high degree of molecular structure is due to extensive hydrogen bonding between glucosidic chains. Local irregularities in the alignment of individual chains are reflected in the structure of the whole fibre, which consists of microcrystalline aggregates separated by amorphous regions. Because of their lesser involvement in hydrogen bonding, glucosidic hydroxyl groups in the amorphous regions are more reactive than hydroxyl groups in the microcrystalline areas of the fibre. Therefore, it would be expected that the chemical derivatization of cellulose would occur preferentially in the amorphous regions. Thus, if one were attempting to substitute hydrophobic

ligands for hydroxyl groups, ligand distribution on the cellulose fibre would be heterogeneous. It should be possible to overcome this heterogeneity through the use of regenerated celluloses, such as rayon, in which the crystalline regions have been removed and the fibre is uniformly amorphous.

We have attempted to introduce hydrophobic ligands into the structure of cellulose in an effort to produce a matrix suitable for the industrial scale immobilization of enzymes through hydrophobic interactions. It was speculated that, if we succeeded in synthesizing hydrophobic cellulose fibres with good enzyme binding capacity, the fibres could be woven into cloth, then rolled and placed into a column to form a spiral reactor. It is reported that a spiral matrix configuration minimizes problems related to substrate and product diffusion to and from the immobilized enzyme. (Vieth and Venkatasubramanian, 1974). The following section describes the extent to which I succeeded in synthesizing hydrophobic cellulose matrices.

Results and Discussion

Four strategies for introducing hydrophobic ligands were tried: 1) epichlorohydrin-mediated linkage of alkyl alcohols to cellulose; 2) epichlorohydrin-mediated linkage of alkyl diols to cellulose; 3) periodate oxidation of

polysaccharide matrices and 4) methylsulphonyl carbanion-mediated matrix derivatization. We have also developed a methodology for appraising the capacity of a matrix to immobilize protein. This procedure, which consists of desorbing matrix-bound bovine serum albumin (B.S.A.) with detergent, followed by a protein assay, is explained in the 'Experimental Procedures' of this chapter.

The first chemical strategy used is outlined in Fig 6, and depends on the susceptibility of epichlorohydrin's epoxide ring to nucleophilic attack by deprotonated hydroxyl groups of either alcohol or cellulose. Attempts were made to link alcohols of various chain lengths to cellulose because solubility problems with very long alkyl alcohols, such as dodecylalcohol, were anticipated. After the derivatization procedure, the treated cotton was washed with organic solvents and water to remove unbound reactants. As shown in Table 5, these fibres bound the same amount of B.S.A. as untreated cellulose. As there appeared to be no change in the physical characteristics (e.g., texture, colour) of the treated fibres, I do not know if derivatization occurred.

The second chemical strategy tried, diagrammed in Fig 7, is similar to the first except that alkyl diols were used instead of alcohols. It was hoped that the use of diols would allow the synthesis of a hydrophobic network around the cellulose fibre. It is conceivable

Fig 6. Epichlorohydrin-mediated substitution of the glucosidic hydroxyl groups of cellulose with alkyl alcohols. 'R' represents a long alkyl chain.

Table 5. Binding capacity of cotton after attempted epichlorohydrin-mediated coupling of alkyl alcohols.

Alcohol	Binding capacity (mg B.S.A./g matrix)
1-octanol	3.0
1-decanol	3.0
1-dodecanol	3.1
Controls:	
untreated cotton	3.1
commercial dodecyl-agarose	40
commercial octyl-agarose	12

Fig 7. Epichlorohydrin-mediated formation of a hydrophobic network around a cellulose fibre.

that an extensive hydrophobic network could greatly increase the binding capacity of the matrix since a single ligand, by virtue of its extreme length, could interact with several protein molecules. At the same time, because the hydrophobic portions of the ligand are interspersed with hydrophilic spacers, the hydrophobicity of the matrix should not be so great as to cause protein denaturation.

The choice of diols used was dictated by their availability. As seen from Table 6, no perceivable increase in protein binding capacity of the treated cotton occurred. In addition, the physical characteristics of the cellulose remained unchanged. As the results of these preliminary experiments involving epichlorohydrin were not encouraging, an entirely different approach was tried, as shown in Fig 8.

The third chemical strategy involved the use of sodium metaperiodate to oxidize adjacent glucosidic hydroxyl groups. Aldehydes generated by oxidation should react with the amino group of alkyl amines, giving rise to Schiff base formation. Subsequent treatment of the cellulose with sodium borohydride would reduce any remaining aldehydes to alcohols, and also reduce Schiff bases to amines. The advantage of this reaction scheme is that the reagents used are safe and relatively inexpensive. A similar strategy was successfully employed by Sanderson and Wilson (1971) to covalently link surface amino groups of B.S.A. to periodate-oxidized

Table 6. Binding capacity of cotton after attempted epichlorohydrin-mediated coupling of alkyl diols.

Diol	Binding capacity (mg B.S.A./g matrix)
1,4-butanediol	3.2
1,5-pentanediol	3.1
Control:	
untreated cotton	3.1

Fig 8. The substitution of polysaccharide matrices with hydrophobic ligands after activation of glucosidic hydroxyl groups with sodium metaperiodate.

polysaccharides.

The substitution reactions were carried out using dodecylamine, since the results of Chapter 3 indicate that 12-carbon chain ligands are suitable for immobilizing enzymes. As seen from Table 7, several oxidation and reduction conditions were investigated in an effort to see if any significant change in protein binding capacity could be detected. It was reported by Sanderson and Wilson (1971) that the extent of protein binding to oxidized polysaccharides was dependent upon the concentration of sodium metaperiodate and the length of polysaccharide exposure to the oxidizing agent. However, I found no evidence that protein binding capacity of cellulose was increased by this chemical treatment.

Matrix derivatization by the fourth chemical strategy met with somewhat more success; the reaction scheme is outlined in Fig 9. In the first step of this procedure, methylsulphonyl carbanion, the conjugate base of dimethyl sulphoxide, was formed according to the method of Corey and Chaykovsky (1962). This highly reactive and very basic carbanion was used to attack and activate glucosidic hydroxyl groups of cellulose. It was anticipated that the resulting sodium alkoxide would react with an alkyl halide to yield cellulose substituted with alkyl chains. As these ligands would be attached to the cellulose matrix through uncharged ether linkages, the immobilization of enzymes to this matrix should be due solely to hydrophobic

Table 7. Protein binding capacity of preparations after oxidation with sodium metaperiodate, exposure to dodecylamine and subsequent reduction with sodium borohydride.

Matrix	Concentration of NaIO_4 (M)	Exposure to NaIO_4 (hours)	Concentration of NaBH_4 (M)	Protein binding capacity (mg B.S.A./g matrix)
cotton	0.5	3	0.1	3.2
cotton	0.5	3	-	3.0
rayon	0.1	3	0.05	1.3
rayon	0.1	12	0.1	1.2
rayon	0.5	3	0.1	1.3
rayon	0.5	3	-	1.3
dextran	0.5	3	0.1	0.4
Controls:				
untreated cotton	-	-	-	3.4
untreated rayon	-	-	-	1.0
untreated dextran	-	-	-	0.5

Fig 9. Methylsulphinyl carbanion-mediated derivatization of polysaccharide matrices.

interactions.

It was found that if the concentrations of bromododecane and sodium hydride were not limited, extensive derivatization of the cellulose matrix apparently took place. This conclusion was based on the observation that the treated cellulose had a waxy and almost oily texture which even exhaustive washing with organic solvents could not remove. It therefore appears that covalent attachment of dodecyl ligands to cellulose had occurred; however, when this derivatized cellulose was soaked in a solution of B.S.A., it was found that very little protein bound to the matrix (Table 8).

In order to investigate the problem of low protein binding capacity, the derivatization procedure was repeated using beads of dextran (Sephadex G-25). Dextran is a glucose polymer, cross-linked with epichlorohydrin to give it rigidity. We therefore thought that dextran might have more uniformly-distributed hydroxyl groups than cellulose. It was found that, when the concentration of the reactants was not limited, the dextran beads were apparently so highly substituted that they floated in water and adhered together to form a sticky mass; the preparation did not bind a significant amount of B.S.A.

The number of methylsulphinyl carbanions generated, and so the number of matrix hydroxyl groups activated, is dependant upon the amount of sodium hydride added in the first step of the reaction. In the above examples,

Table 8. Protein binding capacity of preparations after reaction of methylsulphonyl carbanion-activated matrices with bromododecane.

Matrix	Molar ratio NaH to glucosidic hydroxyl groups ^{a)}	Protein binding capacity (mg B.S.A./g matrix)
cotton	1:1	3.8
rayon	1:1	1.5
rayon	0.2:1	1.4
dextran	1:1	0.5
dextran	0.2:1	0.6
dextran	0.02:1	0.5
dextran	10 ⁻³ :1	0.7
Controls:		
untreated cotton	-	3.2
untreated rayon	-	1.1
untreated dextran	-	0.5

a) See 'Experimental Procedures' of this chapter

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reactant concentrations were such that each hydroxyl group of the polysaccharide matrix could have been substituted with a dodecyl ligand. We postulated that, because of the close proximity of the ligands, hydrophobic interactions between neighbouring dodecyl groups were sufficiently intense as to minimize the possibility of ligand-protein interaction. I therefore attempted to increase the spatial distance between ligands by limiting the concentration of sodium hydride. It was indeed found that the lower the concentration of NaH, the less sticky and buoyant the dextran beads were. However, protein binding capacity remained low.

In retrospect, it appears that the native structure of cellulose and cross-linked dextran (Sephadex G-25) may already be quite hydrophobic, even without the addition of long alkyl ligands. For example, it was reported by Takagahara et al. (1976) that at high salt concentrations proteins bind to cellulose, and are eluted when the salt concentration is decreased. This behaviour is typical of hydrophobic interaction-mediated protein adsorption, as hydrophobic interactions are increased in the presence of neutral salts. Furthermore, Flodin (1962) presents evidence that when a mixture of amino acids is passed through a column of Sephadex G-25, the amino acids are eluted in the order of increasing hydrophobicity. Thus, since the more hydrophobic amino acids are retained longest by the column, it appears that Sephadex G-25

itself is somewhat hydrophobic. If underivatized matrix is hydrophobic, then hydrophobic chains introduced into this structure may fold down and interact with the matrix; these ligands would accordingly not be available for interaction with protein molecules. Therefore, extensive derivatization may lead to intense ligand-ligand interaction, and moderate derivatization to ligand-matrix interactions. Either way, the alkyl chains would not be available for binding proteins. We must therefore conclude that, although cellulose has many attributes which make it an excellent matrix for enzyme immobilization, its usefulness for the immobilization of enzymes through hydrophobic interactions appears limited.

Experimental Procedures

Determination of binding capacity

A known weight of suction-dried matrix was soaked overnight at 37°C in a solution of 0.1 g B.S.A./mL water. In a sintered glass filter, ~~the~~ sample was exhaustively washed with water, then transferred to a known volume of a 10% (w/v) solution of sodium dodecylsulphate (SDS). The mixture was agitated for 30 min, then the matrix was removed and the amount of protein in the solution was determined by the method of Lowry *et al.* (1951). A control experiment indicated that 10% SDS did not interfere with the sensitivity of the protein assay. From the weight of the matrix and the volume of SDS solution used to elute the B.S.A., the protein binding capacity of the matrix could be easily calculated.

Epichlorohydrin-mediated matrix derivatization

In investigating this method of matrix derivatization, three alcohols and two diols were tested. Reactant concentrations were as suggested by Peterson and Sober (1956) in a paper describing the coupling of triethanolamine to cellulose. The following reaction mixture was prepared in five separate vials: 9.0 mL 10 N NaOH, 3.6 mL epichlorohydrin, 2.0 mL of alcohol or diol and 1.0 g cotton. Vials were vortexed and left overnight at 25°C. The reaction was terminated by washing the

matrices with several portions of ether-ethanol (1:1,v/v), and then with ethanol. This was followed by exhaustive washing with water, and then determination of protein binding capacity.

Periodate oxidation of matrices

Matrix was soaked for various lengths of time (generally three hours) in a freshly prepared, unbuffered solution of NaIO_4 ; it was then exhaustively washed with water in a sintered glass filter. The suction-dried material was placed in a saturated solution of dodecylamine (pH 7.0) for 3 hours, and then transferred to a freshly prepared solution of NaBH_4 , pH 8.0, for one hour. The reaction was terminated by washing the matrix with organic solvents and water, as above.

Methylsulphonyl carbanion-mediated matrix derivatization

Since NaH reacts violently with water, all reactants were dried prior to use: matrices were heated at 120°C overnight, then kept in a dessicator, and DMSO was kept over molecular sieves. The exclusion of water from the system also ensured that all carbanions generated would react with hydroxyl groups on the matrix, and not with water. The degree of ligand substitution on the matrix could apparently be controlled by the concentration of NaH . An estimate of the amount of NaH added to DMSO which would give rise to one carbanion per glucosidic hydroxyl

group was calculated as follows. A polysaccharide such as cellulose is composed of glucose subunits. However, the formula weight of each subunit is not 180, as in the case of glucose, but 162, since a water molecule is eliminated when glucose subunits link together. Therefore, a 'mole' of polysaccharide glucose subunits weighs 162 g. If it is assumed that no hydrogen bonding takes place between glucosidic hydroxyl groups, then each glucose subunit contains 3 hydroxyl groups which can be activated by reaction with methylsulphonyl carbanion. From these assumptions, one gets a rough estimate as to the moles of hydroxyl groups present in a given weight of polysaccharide.

The reaction was carried out in a small beaker containing about 10 mL DMSO. Through the use of a combination block heater-magnetic stirrer, the contents of the beaker were continuously agitated and maintained at 70°C. Once this temperature was achieved, about 0.1 g dried matrix was added, together with the appropriate amount of NaH. Once hydrogen gas evolution ceased (about 20 min) an excess of 1-bromododecane was added and the mixture was left stirring for one hour. Throughout the reaction, the beaker was kept covered in an effort to further eliminate water from the system. The reaction was terminated by washing the matrix with organic solvents and water.

GENERAL DISCUSSION AND CONCLUSIONS

Two questions of Biotechnological orientation have been addressed in this thesis. The first question was: how can cloned, hybrid penicillinase-mammalian gene products be efficiently extracted and assayed? When a gene specifying a mammalian peptide is fused to a plasmid penicillinase gene, the synthesized peptide does not suffer proteolysis, since the peptide is exported to the bacterial periplasmic space. This is advantageous from a commercial view-point, as the yield of the eukaryotic protein would probably be higher than if it remained within the cytoplasm. Furthermore, as the number of protein species is fewer in the periplasmic space than in the cytoplasm, the exportation of the peptide would facilitate its purification. Thus, an assay specific for penicillinase would allow the accurate determination of this enzyme's activity; this is important as penicillinase activity reflects the concentration of the cloning vehicle on which the penicillinase and mammalian genes are carried. In addition, a rapid and inexpensive means of extracting the hybrid protein would help reduce the cost of producing the peptide on a commercial scale.

We introduce here a penicillinase assay which is specific for the enzyme. Unlike the commonly used iodometric methods, this assay can be used on either cell-free, aqueous extracts of the enzyme, or on crude,

permeabilized cell cultures. Thus, it is no longer necessary to extract penicillinase from bacteria in order to assay its activity. We further present a rapid, yet efficient procedure for extracting periplasmic penicillinase from E. coli. The new method extracts the same amount of penicillinase activity from a bacterial culture as the more involved, conventional osmotic shock procedure (Table 1). Evidence is presented that the PEA-EDTA method extracts periplasmic proteins in general with the same degree of efficiency and specificity as osmotic shock. Thus, our extraction procedure has not only potential in the event of commercial production of cloned eukaryotic peptides exported to the bacterial periplasmic space, but may also find applicability in numerous laboratory studies.

The second question addressed in this thesis was: can hydrophobic interactions provide a gentle, yet effective means of immobilizing enzymes? When five enzymes of potential or proven commercial value were exposed to dodecyl-agarose, all five formed active immobilized enzyme preparations (Tables 3 and 4). All but one of the preparations showed continuing good performance over time. As dodecyl-agarose is unacceptable as an enzyme matrix in industrial reactors, several attempts were made to synthesize inexpensive, durable, hydrophobic derivatives of cellulose. Although at least one of the reaction schemes apparently succeeded in introducing alkyl chains

into the cellulose backbone, none of the cellulose matrices bound a significant amount of bovine serum albumin. One possible reason for the low binding capacity is that cellulose itself may be quite hydrophobic, and interacts strongly with the ligands covalently-bound to it. Although success in synthesizing cellulose matrices for enzyme immobilization through hydrophobic interactions was limited, my work has nevertheless provided the framework for numerous other enzyme immobilization procedures presently being investigated in this laboratory.

APPENDIX

A short study into the action of p-aminobenzamidine on the maturation of bacterial periplasmic proteins

Introduction

As explained in Chapter 2, newly-synthesized immature forms of periplasmic proteins, still within the cytoplasm, have an additional 15-30 amino acid residue sequence on the amino terminal end of the peptide which is absent from the periplasmic form of the protein. While several hypotheses attempt to explain the role of the leader sequence in the secretion process (Halegoua and Inouye, 1979; Wickner, 1979; Emr et al., 1980), there has been little speculation on the actual mode of the maturation process. In particular, it is totally unknown which enzymes act to cleave the leader sequence from the protein proper. This appendix describes an attempt to identify the protease responsible for protein maturation.

MacGregor et al. (1979) have reported that the addition of the protease inhibitor p-aminobenzamidine (pABA) to a culture of growing E. coli cells results in the accumulation of immature outer membrane proteins, but does not affect the biosynthesis of cytoplasmic membrane proteins. These results suggest that pABA might specifically inhibit the maturation of proteins being

excreted across the cytoplasmic membrane. If this is the case, then pABA should cause the immature forms of periplasmic proteins such as penicillinase to accumulate. MacGregor et al. (1979) did not hypothesize why pABA causes immature protein accumulation. However, it is known that this chemical is a non-hydrolyzable analogue of arginine and a reversible trypsin inhibitor (Mares-Guin and Shaw, 1965); i.e., the greater the ratio of pABA to trypsin, the more evident is the inhibition of the enzyme.

In the following pages, we tentatively speculate as to the mode of action of pABA on the maturation process of periplasmic proteins. As discussed in Chapter 2, the amino acid sequences of eight prokaryotic leader sequences are known (Emr et al., 1980; Inouye and Halegoua, 1980). The sequences are all similar in that they have an amino-terminal hydrophilic region and a carboxy-terminal hydrophobic area. Without exception, the last hydrophilic amino acid, before the start of the hydrophobic sequence, is either lysine or arginine. This fact suggests that one of the proteases responsible for the cleavage of the leader sequence from the remainder of the protein has a trypsin-like specificity. If one considers the similarity in the structure of signal sequences, together with the fact that there are several hundred different periplasmic and outer membrane proteins (all of which must traverse the cytoplasmic membrane), then the logic of having only a few types of proteases to process all these proteins

becomes obvious. It would be unwieldy for the bacterium to require a unique protease to process each type of excreted protein. We suggest that only two proteases are necessary to process all extra-cytoplasmic proteins. A leader sequence could be first cleaved on the carboxy side of the lysine or arginine residue by a trypsin-like endo-peptidase. This would remove the hydrophilic portion of the leader sequence. The remainder of the sequence might then be digested by an exo-peptidase specific for hydrophobic amino acids. I describe below our attempts to obtain experimental evidence for the role of a trypsin-like enzyme in the maturation process of bacterial periplasmic proteins.

Results and Discussion

If such a trypsin-like processing enzyme is involved in maturation, then pABA should cause the accumulation of immature periplasmic proteins as well as the outer membrane proteins observed by MacGregor et al. (1979). In order to test our hypothesis, WA802/pBR322 was again employed. If penicillinase maturation is prevented by the inhibitor, we would expect the cells to gradually become sensitive to ampicillin, as immature penicillinase dilutes out the mature, periplasmic form of the enzyme. MacGregor et al. (1979) observed immature outer membrane protein accumulation when bacterial cells were grown in 15 mM

pABA. We found that this concentration of inhibitor in a culture of WA802/pBR322 had an immediate, but mild, inhibitory effect on bacterial growth; normal growth was resumed within one doubling time (Fig 10). However, whereas an untreated culture of WA802/pBR322 was totally unaffected by the presence of even milligram quantities of ampicillin per millilitre of culture, the bacterial strain immediately became extremely sensitive to the antibiotic upon exposure to 15 mM pABA (Fig 10). This effect, although severe, was transitory; the cells eventually recovered and resumed normal growth. The suddenness and severity of this effect might be explained by pABA inhibiting penicillinase maturation at the site of bacterial division. A lack of active penicillinase in this area of rapid cell-wall synthesis would be especially detrimental to the cell.

E. coli is quite sensitive to the concentration of pABA in the growth medium. Both WA802/pBR322 and CA8000, a wild-type strain, exhibited increased growth inhibition with increasing pABA concentrations; at levels above 30 mM pABA cell growth stopped entirely and did not resume. By following the incorporation of a ¹⁴C amino acid label into trichloroacetic acid (TCA) insoluble material (Fig 11), it was found that bacterial protein synthesis ceased within 8 min of exposure to 30 mM pABA. Therefore, pABA at high concentrations appears to indiscriminately inhibit all protein synthesis. However, judging from the reversible

Fig 10. The effect of 15 mM pABA on the growth of WA802/pBR322. Cells were grown in TMM supplemented with glucose, phosphate and methionine to log phase. The average doubling time of untreated cells was 60 min. Once log phase was achieved (arrow), one portion of the culture was treated with a final concentration of 15 mM, pH-neutralized pABA, while another portion was simultaneously treated with 15 mM pABA and 200 μ g/mL ampicillin. Symbols: ($\square$), untreated culture plus or minus 200 μ g/mL ampicillin; ($\circ$), culture treated with 15 mM pABA; (Δ), culture treated with 15 mM pABA and 200 μ g/mL ampicillin.

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Fig 11. Incorporation by CA8000 of ^{14}C -lysine into TCA insoluble material in the presence and absence of 30 mM pABA. Symbols: ($\square$), cpm from ^{14}C -lysine incorporated into 30 mM pABA treated cells; ($\circ$), cpm from ^{14}C -lysine incorporated into untreated cells.

7 effect of the drug in causing ampicillin sensitivity, pABA at lower concentrations may act more specifically to inhibit only protein maturation.

Since bacterial cells eventually recover from the effects of lower levels of pABA, it is possible that the accumulation of immature proteins triggers the induction of the speculated trypsin-like maturation protease. A higher concentration of the protease would help overcome the inhibitory effect of pABA. Protease induction would be a simple means by which a bacterium could avoid a potentially lethal situation, since the complete halt of protein exportation must eventually lead to cell death.

Support for this hypothesis was obtained by growing CA8000 in 15 mM pABA until the cells recovered and resumed normal growth. The culture was then diluted with fresh growth medium and split in half. Half the culture was immediately re-treated with 15 mM pABA; the other half was first allowed to grow for 1.5 doublings before the inhibitor was re-introduced. As seen from Fig 12, the growth rate of bacteria immediately re-treated with the inhibitor was unaffected by pABA, whereas when the inhibitor was added back to the culture which had been without pABA for over an hour, a transitory decrease in growth rate was again observed. These results are consistent with the hypothesis that a protease is induced by the inhibitory effect of pABA, and that this induction acts to overcome the effect of the inhibitor. Induced

Fig 12. The effect of multiple pABA treatments on the growth of CA8000. Cells were grown in TMM supplemented with glucose, phosphate and thiamine to log phase. The average doubling time of untreated cells was 60 min. Once log phase was achieved, the culture was treated with pH-neutralized pABA to a final concentration of 15 mM (arrow 1; symbol $\circ$). After the cells had overcome the effect of the inhibitor, the culture was diluted with fresh growth medium. Part of this diluted culture was immediately re-treated with 15 mM pABA (arrow 2; symbol Δ), whereas part was allowed to grow for 1.5 doublings before the re-introduction of the inhibitor (arrow 3; symbol $\square$).

cells remain insensitive to pABA as long as the inhibitor is present in the medium. If pABA is removed and then later re-introduced, the cells become sensitive again and growth is transiently affected.

To test the above hypothesis directly, attempts were made to measure trypsin-like protease levels in both untreated CA8000 cells, and in cells which had recovered from 15 mM pABA. Two artificial trypsin substrates were tried: benzoyl-DL-arginine p-nitroanilide (BAN) (Erlanger et al., 1961) and N-benzyl-L-arginine ethyl ester (BAEE) (Keszdy et al., 1965). Since the protease would likely be either cytoplasmic or bound to the inner membrane, attempts were made to make the cell membrane permeable to these protease substrates. Several methods were tried, including lysozyme and detergent treatments. However, when permeabilized cells were added to a 5 mM solution of BAN, the substrate promptly precipitated out. A small amount of substrate must have remained in solution since gradual substrate hydrolysis became evident after several days. Furthermore, cells which had recovered from pABA treatment appeared to hydrolyze the substrate slightly faster than untreated cells. However, the rate of hydrolysis was so slow that a meaningful determination of bacterial protease activity was impossible. Purified trypsin, on the other hand, immediately hydrolyzed all BAN remaining in solution.

We were unable to detect any hydrolysis of the second substrate, BAEE, by either permeabilized cells or purified trypsin. Therefore, due to difficulties encountered assaying the enzyme, we have not obtained experimental evidence to support the theory that bacteria which have recovered from pABA treatment contain higher levels of a trypsin-like protease than untreated cells. Nevertheless, the hypothesis presented above suggesting the role of a trypsin-like endopeptidase in the maturation of immature bacterial proteins is a possible and simple explanation for a process deserving of inquiry but as yet unstudied.

Experimental Procedures

¹⁴C-Lysine incorporation into TCA-insoluble material

CA8000 was grown in TMM supplemented with glucose, phosphate and thiamine to early log phase ($A_{500} = 0.4$). At '0' time, 2 X 1 mL aliquots of culture were removed. One aliquot was mixed with 1 μ Ci ¹⁴C-lysine, 10 μ g lysine and pH-neutralized pABA to a final concentration of 30 mM. The other aliquot of culture was added to a similar ¹⁴C-lysine brew, except that water substituted for pABA. Immediately after vortexing, 50 μ L were removed from each tube and spotted on individual, numbered Whatmann No. 3 MM paper disks; disks were dried in a stream of cold air for 20 sec, then dropped into ice-cold 10% (w/v) TCA. The remainder of the labelled cultures were shaken at 37°C. At staggered 1 min intervals, the process of spotting 50 μ L aliquots of culture on paper disks, drying and treating with 10% TCA was repeated. This procedure was carried out for 30 min. All disks were left in the ice-cold 10% TCA for at least half an hour; they were then transferred to 5% (w/v) TCA at 80°C for a further 30 min. The disks were then rinsed 3 times with ice-cold 5% TCA, and 3 times with ether-ethanol (1:1, v/v). After drying, each disk was placed in a scintillation vial and counted in a liquid scintillation counter.

Assay of intracellular trypsin-like proteases

CA8000 was grown as above to $A_{500}=0.4$. Half the culture was treated with 15 mM pABA. By the time the cells had reached $A_{500}=1.0$, they had recovered from the effects of the inhibitor. Several 1 mL portions of both the treated and untreated cultures (also at $A_{500}=1.0$) were filtered and resuspended in 0.5 mL of a 1 mg/mL lysozyme solution in 0.05 M Tris-HCl and 5 mM EDTA, pH 8.2. All samples were frozen and thawed once to help weaken the cell walls. One of each of the following compounds at a final concentration of 1% was added to both a pABA treated and untreated sample: sodium deoxycholate, Triton X-100, toluene, phenethyl alcohol, Brij 58 or N-lauryl sarcosine. BAN, dissolved in dimethylsulphoxide, was added to a final concentration of 5 mM; samples were then incubated at 37°C for several days. The extent of BAN hydrolysis could be determined by measuring the yellow colour due to p-nitroaniline at 410 nm. The extinction co-efficient of p-nitroaniline is 8800 litres/mole/centimetre.

A similar experiment was carried out to see if pABA treated and untreated CA8000 permeabilized cells would cause the hydrolysis of BAEE. Substrate was added to a final concentration of 1 mM. Attempts were made to measure, over several days, the hydrolysis of BAEE by following changes at 255 nm, and assuming a molar extinction co-efficient of 808 litres/mole/centimetre.

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