Journal of Chromatography, 473 (1989) 1–26
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 473

REVIEW

APPLICATION OF ENZYME PURIFICATION PROCESSES TO PROTEOLYTIC ENZYMES

CHANTAL BARTHOMEUF

Laboratoire de Pharmacognosie et de Microbiologie Industrielle, Faculté de Pharmacie, Université de Clermont–1, 28 Place Henri-Dunant, B.P. 38, 63001 Clermont-Ferrand Cédex (France)

(First received February 6th, 1989; revised manuscript received March 7th, 1989)

CONTENTS

0021-9673/89/$03.50

INTRODUCTION

The proportion of proteolytic enzymes in the chain production of industrial enzymes is 60%, that is, *ca.* 40 000 tons per annum. Interest in these enzymes is increasing. In addition to their traditional applications, such as in industrial detergents, new outlets are opening up, *e.g.*, in the production of polypeptides, as they permit new functional or nutritional properties to be introduced and biologically active peptides to be obtained. Further, their use in the field of biosynthesis is now expanding considerably.

Purification methods vary according to the chosen purpose, *e.g.*, preparation of industrial enzymes or standards for the determination of enzymatic activity or for structure analysis.

A decrease in overall activity occurs during the various stages of purification. Hence one must aim to obtain a pure enough enzyme for the intended purpose so that the cost/performance ratio is as small as possible.

As enzymes are more or less unstable, care must be taken in the extraction and purification steps: (i) operation at low temperatures hampers the development of microorganisms, limits the enzyme–substrate reaction and prevents degradation of the thermolabile enzymes; nevertheless, as some proteases are fairly stable at high temperature, this property is used in order to eliminate the thermolabile contaminants; and (ii) addition of antiseptics, antibiotics, reversible inhibitors of enzymatic reactions, such as EDTA, soybean trypsin inhibitor, pepstatin or stabilizing factors such as calcium ions. It is also desirable to avoid denaturing conditions such as extremes of pH, too high ionic strength or addition of surface-active or organic solvents. Foams and films should be avoided whenever possible, as proteins tend to denature on interfaces.

Three main steps can be distinguished in an enzymatic preparation: extraction of endocellular enzymes and solubilization in the medium; obtaining the crude enzyme; and selective purification, and these are treated in the following sections.

1. EXTRACTION OF ENDOCELLULAR ENZYMES AND SOLUBILIZATION IN THE MEDIUM

Before any separation, the enzyme must be solubilized in the medium and separated from insoluble contaminants such as microorganisms, cellular debris or various impurities.

The endocellular enzymes or those obtained from tissue extracts are dissolved after membrane disruption and homogenized in a buffer. The membrane disruption is carried out by various chemical methods, *e.g.*, alkalinization, addition of EDTA or detergents or osmotic shocks, or by physical methods, *e.g.*, sonication, freezing and thawing phases, solid or liquid shear or grinding or agitation with abrasives[1]. In a number of instances, an important part of the enzyme remains adsorbed within the cellular membranes.

Insoluble impurities and cellular waste are then removed by filtration, centrifugation or partition. With conventional methods, because the very small size of the particles which are to be eliminated, there is a risk of plugging, entailing losses or a decrease in activity. Tangential filtration may be a solution.

In addition to the classical methods used to extract enzymes when prepared in suitable amounts, partition techniques are more specially used for expensive enzymes. Hence liquid–liquid extraction is being increasingly used as it permits not only cells and insoluble organelles to be eliminated, but also the enzymatic activity of poorly concentrated media such as fermentation juices or filtrates from cellular cultures[2] to be increased. It is based on the incompatibility in an aqueous solution of two polymers [polyethylene glycol (PEG) and dextran] or of one polymer and an appropriate salt (principally potassium phosphate), which leads to the formation of two non-miscible phases. The two phases contain a high proportion of water (65–90%), which ensures a better enzymatic stability than water–organic solvent mixtures[3].

For each enzyme, one can determine a partition coefficient specific for a given medium, $K = C_T/C_B$, where C_T is the concentration of enzyme in the upper phase and C_B that in the lower phase. For a high degree of purification, the enzyme should have a large (>3) or small ($<1/3$) K value. K is a constant and depends on numerous factors such as the type of polymer being used, molecular weight and molecular weight distribution of the polymers, length of the tie-line (a function of the concentration of the components of the phase system), type and concentration of added salts, pH and temperature[4]. Hydrophobic proteins are usually found in the upper phase and hydrophilic proteins in the lower phase. Enzymes can be extracted into the chosen phase by introducing a specific ligand bound to a polymer concentrated in such a phase[2,5,6]. The output depends on the partition factors and on the relative volumes of the two phases. Enrichment can be carried out in several stages. It is important to know precisely the point of equilibrium and the phase separation method. It is also possible to use three or poly-phase systems; these are advantageous compared with two-phase systems as mixtures of proteins can be resolved between three or more bulk phases. The possibility of binding specific ligands to each of the polymers could lead to a further increase in the selectivity of partitioning[7].

The formation and separation of the phases, recycling of polymers and salts and economic implications have been thoroughly studied by Husted and co-workers[8–11]. The possibilities of continuous output (counter-flow extraction) enables both high yields (50 g proteins/kg medium) and high purities to be obtained. This non-

denaturing technique is fairly recent and apparently seldom used for proteases, but it should be developed more. At present, its industrial application meets with some difficulties regarding the recovery of the various polymers in order to recycle them.

2. OBTAINING THE CRUDE ENZYME

At the beginning of a separation, when large amounts of materials have to be handled, methods of high capacity should be chosen; at this stage resolution is secondary requirement. We can distinguish precipitation techniques and adsorption techniques, and these are consisted below.

2.1. Precipitation techniques

These are likely to be used directly on the starting medium, which has previously been treated to remove insoluble contaminants subjected to a preliminary concentration step.

2.1.1. Precipitation with organic solvents

Organic solvent-induced precipitation (2–5 volumes) is a still in use as a classical method, which nonetheless has some inconveniences, particularly because of the risk of denaturation. In addition, it requires the use of large amounts of solvents, especially for low-molecular-weight enzymes. This makes it necessary to treat large amounts and requires a solvent-recovery apparatus to be installed.

2.1.2. Precipitation with neutral salts

Salting-out of proteins is based on the property of proteins to re-precipitate at a high ionic strength. A protein solution of known concentrations (s) will begin to precipitate at a ionic strength given by the following equation:

$$\frac{T}{2} = \frac{B_1 - \log s}{K_1 s}$$

where T = ionic strength (mol/l), B_1 = constant, $K_1 s$ = (slope of line) salting-out constant and s = solubility of protein (g/l). The value of B_1 is dependent of the salt used and varies with pH, temperature and the nature of protein in solution. $K_1 s$ is independent of pH and temperature but varies with the protein in solution and the salt used[1]. One usually operates at the enzyme pI. One can possibly obtain a fractionation of the medium by using an increasing concentration of salt. The most commonly used salt is ammonium sulphate because of its high solubility, lack of toxicity, cheapness and, in some instances, its stabilizing effect.

In this method, however, the mineral salts are liable to be carried with the precipitate, which requires another step such as dialysis or gel filtration.

Both methods of precipitation have the advantages of being simple, cheap and able to treat large amounts of impure product, but they are hardly applicable to enzymes of low molecular weight because of their high solubility in water.

2.1.3. Precipitation with non-denaturing polymers

It is also possible to utilize non-denaturing polymers such as polyethylene glycol

(PEG). They are used alone or associated with ethanol or ammonium sulphate[12]. The great advantage of this process is the shorter time required for precipitation (0.5–1 h). Other advantages are in aiding crystallization and limiting enzyme degradation. The most commonly used PEG have a nominal average molecular weight (MW) in the range 4000–6000 but polymers with much lower MW, which are cheaper, can also be used. It is advisable to buffer the PEG solution at the required pH in order not to change the pH of the enzyme solution, which might lead to conditions that promote molecular association[12]. The PEG is then removed by ultrafiltration or during the later stages of ion-exchange or affinity chromatography[13]. As PEGs are optically transparent and help to prevent enzyme loss, it is unnecessary to eliminate the last traces[14].

2.1.4. Particular instances

2.1.4.1. Precipitation with metals

The use of bivalent metals such as zinc or copper to precipitate proteins has been employed for many years[15–17]. These metals form relatively stable complexes with histidine and cysteine residues[18–20], which permits the proteins to be precipitated without denaturation by reducing the pH variations and the amounts of solvents required in classical systems. Zaworski and Gill[21] have recently published a method using Zn^{2+} for the rapid concentration of culture media and obtained a porcine urokinase from *Saccharomyces cerevisiae* culture filtrates.

2.1.4.2. Affinity precipitation

The principle consists in complexing the enzyme and precipitating the complex macroligand–molecule. This technique seems very promising as it can be applied on a large scale. Schneider *et al.*[22] produced an acrylamide water-soluble macroligand carrying affinitive molecules for trypsin and benzoic acid that helps its precipitation under certain conditions. As the ligand can easily be recycled, this technique is attractive, it is cheap and it can be applied to large volumes, although it is sometimes difficult to find a suitable ligand. This method is, moreover, fairly recent and has rarely been applied to proteases.

2.2. Adsorption techniques

An alternative to precipitation is selective adsorption of the enzyme on an appropriate support.

2.2.1. Adsorption on an ion-exchange resin

This is the most interesting process, especially when a high-capacity and inexpensive exchanger can be used and/or when batchwise operation is possible. Under precise conditions of pH, this can be a highly selective method.

Whenever the enzymes are fragile or do not withstand high ionic strengths, operating in the reverse mode is possible, *i.e.*, fixing the contaminants on the resin, then concentrating the enzyme.

2.2.2. Adsorption on specific ligands

In this stage, affinity-related chromatography is usually too expensive except for use with a highly active product. It is used for the purification of expensive enzymes.

3. SELECTIVE PROCESSES OF PURIFICATION

The capacity and selectivity of the methods are dependent on the amount of contaminants, the volume to be dealt with and the purity required. On an industrial scale, it is advisable to limit the number of stages. Two types of techniques can be singled out: chromatographic and electrophoretic.

3.1. Chromatographic techniques

3.1.1. Low-pressure liquid chromatography

The choice of the chromatographic support must meet certain requirements, such as specific adsorption, non-denaturation of adsorbed products and perfect reversibility of the adsorption. Other criteria should also be considered, such as the adsorption capacity of the support, the usable flow-rate range, chemical stability and yield–cost ratio[23].

3.1.1.1. Ion-exchange chromatography

This widely used method has a number of advantages: high capacity, good specificity depending on the operating conditions and inexpensive supports. One can distinguish two main type of materials, ion-exchange resins and hydrophilic cellulosic ion exchangers.

Ion-exchange resins are insoluble polymers on which the exchange groups are fixed. Resin have the advantages of being stable over a wide range of pH, they sediment rapidly, they have a high adsorption capacity and high liquid flow-rates can be used. They are commonly used in industrial partitioning (Zeta-prep, LKB), but their high capacity is often disadvantageous for analytical purification as enzymes do not withstand the harsh conditions necessary for their elution.

The chief hydrophilic cellulosic ion exchangers on the market and their properties were given by Segal[24].

The choice of the exchanger depends on the sample properties (charge, pH, stability), the amount to be treated and the level of purity required. The net charge of the molecule determines the type of exchanger. Usually, one uses weak exchangers in the pH range 6–9 and strong exchangers for slightly ionized substances eluted at extreme pH or with a high ionic strength. The nature of the matrix, the height/diameter ratio and the flow-rate are determined according to the required level of purification. On an industrial scale, one usually selects a height/diameter ratio of *ca.* 15–18. The enzyme is then eluted with a gradient of pH or a gradient of ionic strength with or without a pH gradient; stabilizing agents and/or enzymatic reaction inhibitors can be added.

3.1.1.1.1. Chromatofocusing. This is ion-exchange chromatography of a particular kind, developed by Sluyterman and Wijdenes[25–27], which utilizes the elution capacity of a weak cation exchanger. The eluent consists of a mixed buffer of various charged components (polybuffer). At the start, the column is stabilized at the highest value of the chosen pH gradient and the polybuffer is at the lowest pH. A pH gradient is then obtained automatically as elution proceeds, which permits focusing, *i.e.*, proteins will move along the column at or near their p*I* values. Flow-rates even lower than 0.5 ml/min are useful. The gradient slope is controlled by the concentration of

polybuffer being pumped into the column. The two types of column commonly used are Pharmacia Mono P and the Synchrom AX series. This simple, fast technique enables sizeable amounts (0.5–5 mg) of enzymes to be recovered without denaturation. It is particularly suitable for the separation of similar molecules such as isoenzymes.

3.1.1.2. Gel filtration chromatography

This method, also called exclusion or gel permeation chromatography, is well established. The molecules are eluted in order of decreasing molecular weight. The working range is chosen according to the purity desired, the molecular weight range and the concentration of contaminants. A summary of the different gels on the market was published in *Biofutur*[24]. The most commonly used are the Sepharose G series (Pharmacia) and the Bio-Gel P series (Bio-Rad Labs.). For MW above 100 000 daltons, Agarose gels, Sepharose (Pharmacia) and Bio-Gel A series (Bio-Rad Labs.) are currently used.

When the proteases for purification are metallo-enzymes, it is often necessary to maintain the integrity of the metal–enzyme complex during the chromatographic separation, for example to study enzyme activity. In order to avoid loss or substitution of metal ions, it may be necessary to operate on a metal-free chromatographic medium. The mode of preparation of such media and of determination of metal binding capacity were detailed by Martin[28].

3.1.1.3. Hydrophobic interaction chromatography (HIC)

Enzymatic separations on alkyl-agarose (Sephadex C*n*) columns depend on the formation of hydrophobic complexes between the hydrophobic amino acid areas of the enzyme and a ligand (X) covalently linked to an inert matrix on interposing a hydrocarbon chain (an "arm"). It may be possible to modify the intensity of the interaction by modifying the length of the arm. The X groups can be NH_2, COOH, OH, I, $CH=CH_2$, $-C\equiv CH$ or $-CH(CH_3)_2$. The commonest are the NH_2 groups[29]. The association occurs mainly when the medium has a high strength, this technique therefore being especially suitable for samples obtained from a saline precipitation. Nevertheless, as the elution conditions are sometimes drastic, the method is limited to particular cases. Proteases purified by this process include procarboxydase A and B[29], collagenase[29], kininase II[30], renin[31] and hornet chymotrypsin[32]. The selection of the column for a given precipitation, the preparation of column materials and the optimization of separations were studied by Shaltiel[29].

3.1.1.4. Adsorption chromatography on mineral polymers

The most commonly used material is hydroxyapatite. This crystalline calcium phosphate, $Ca_{10}(PO_4)_6(OH)_2$, permits a rapid fractionation of enzymes. The separation process involves the secondary and tertiary structure of the molecule, the neutral and acidic proteins apparently being fixed on the Ca^{2+} sites and the basic proteins on the PO_4^{3-} sites[1].

A great advantage of chromatography on hydroxyapatite is that it can be used with phosphate buffer at a pH close to the physiological pH and be virtually independent of the initial saline concentration of the sample, *i.e.*, it permits elution through a simple increase in phosphate concentration (usually 30–120 m*M*). The resolution depends on the shape, charge and charge/mass ratio. This technique is

particularly suitable for purifying labile enzymes and for the concentration of samples obtained by ion-exchange chromatography that have possibly been chromatographed without previous dialysis. The main inconvenience is the very low flow-rate[33]; different workers have proposed various methods to improve this[34]. Moreover, commercially available supports with shapes suitable for preparative chromatography, such as Bio-Gel HT and HTP (Bio-Rad Labs.) and HA-Ultragel (IBF) help to overcome the difficulties with the technique.

3.1.1.5. Affinity chromatography

Affinity chromatography is an adsorption chromatographic technique in which the molecule to be purified is selectively and reversibly adsorbed on a biospecific ligand. In some instances, the purification was significantly improved by interposing an "arm" which relieves the steric restrictions imposed by the matrix and allows an increased flexibility and availability of the ligand. The main coupling methods and their advantages and inconveniences were outlined by Scopes[35]. After eliminating the contaminants by washing, the enzyme is eluted by pH modification, a salt gradient, specific eluents, distorting eluents (urea) or by reducing the adsorbent polarity.

We can distinguish specific ligands, usable with similar enzymes from different origins, the main proteolytic ligands being listed in Table 1, and group ligands, commonly related to a variety of enzyme. The main ligands for serine proteases can be listed as follows.

The most commonly used aldehydic peptides[36,37] are the arginal derivatives, which show a very strong affinity for trypsin-family enzymes. Enzymes eluted from Argal–Sepharose (4B or 6B) are prekallikrein[38], *Clostridium histolyticum* clostripain[39], human plasminogen activator[40], prothrombin[41], Pronase (mixture of various proteases produced by *Streptomyces griseus*[36]), bovine trypsin[36] and mixtures of trypsin and chymotrypsin[37].

Soybean–trypsin inhibitor[42,43]: the Kunitz soybean inhibitior is frequently used to purify proteases, *e.g.*, trypsin, chymotrypsin[44], crab collagenase or kallikrein[45]. A few proteases can be separated with the aid of the Bowman Birk soybean inhibitor[46].

The benzamidine group has been used for rapid separations of numerous proteases, trypsin[47], thrombin[47], urokinase[48], prekallikrein[49], kallikrein[50,51], collagenase and clostripain[52] or second component of human complement. It is possible to utilize the benzamidine group to detect specifically active forms of some serum proteases such as trypsin, thrombin or human factors IXa and Xa[53].

Phenylboronic acids are used in the separation of trypsin and chymotrypsin[54] and of different subtilisins[55].

A recent technique that makes use of a new isopropyl fluorophosphate gel is of great interest in the separation of serine proteases[56].

The main ligand for the aspartate proteases is the hexapeptide Val–D–Leu–Pro–Phe–Phe–Val–D–Leu[54] and for thiol proteases some peptidyl diazomethyl ketones, which are more selective. They react rapidly at high dilution with thiol proteases but not with other proteases and can be synthesized to satisfy the specificity of individual members of the thiol protease family[57]. However, in the presence of Cu^{2+} ions, certain proteases can be inactivated[57].

As most ligands are inhibitors, research has centred on new inhibitors specific to the enzymes being investigated, such as α_2-macroglobulin, human α_1-proteinase

inhibitor, human α_1-antichymotrypsin, eglin (elastase–cathepsin G inhibitor from leeches), egg white cystatin and potato carboxydase inhibitor (CPI)[58]. Because CPI fixes numerous proteases, it is particularly interesting in the field of affinity chromatography[59]. It is stable at extremes of pH, not denatured by guanidine–6 *M* HCl and can be coupled to Sepharose. It has been used to separate various proteases, such as bovine and porcine carboxypeptidases A and B, carboxypeptidase N and cathepsin[59,60].

3.1.1.5.1. Affinity chromatography on immobilized dyes. Dyes, especially Cibacron Blue F3G-A, appear to be especially effective adsorbents for the purification of numerous proteases, including clotting factors, complement factors and human hexokinase[61]. Other dyes, such as Procion Green H-4G and H-8BN, have also been found to be suitable ligands for a yeast hexokinase[62] and carboxypeptidase G_2, respectively[63]. The mode of action is not well known but there seem to be interactions of the ion-exchange type and/or hydrophobic interactions[61]. This method is advantageous in several respects, being cheap and applicable to coupling, chemical and enzymatic studies, which makes it useful for large-scale purifications, but it lacks specificity. Several techniques for such dye-screening procedures have been published[35,61]. It must be operated under well defined buffer, temperature and pH conditions. The eluents can be non-specific, *i.e.*, molarity, salts, polyols, chaotropic or chelating agents and detergents, or specific, *i.e.*, molarity, substrates and dyes.

3.1.1.5.2. Lectins. When the enzymes are glycoproteins, which is frequently the case for proteases, the use of lectins as ligands is very helpful. The specificities of the main lectins have been reported by Segal[64], the more frequently used being concanavalin A (Con A), which recognizes molecules whose glucosylated part includes D-mannose or D-glucose. Lectins have numerous advantages as they are very selective molecules, available in large amounts. Also, as they do not interact very strongly with sugars ($K = 10^2$–10^4), they can be displaced easily by specific sugars at neutral pH and separated by concentration gradients[65]. Lectins are generally used in the early stages of purification and require additional procedures for complete purification; details of specific methods are not considered. The general method for their use has been reviewed by Lotan and Nicolson[66].

3.1.1.5.3. Immunoaffinity chromatography on antibody columns. Chromatography with antibodies or more recently with monoclonal antibodies has been used to separate some therapeutic proteases such as kallikrein[67], angiotensin I-converting enzyme[68], proteolytic enzymes of the complement system[69–71], blood-clotting enzymes[72–74] and urokinase[75].

3.1.1.5.4. Metal ion affinity chromatography (IMAC chromatography). This technique makes use of the capacity of metals to coordinate molecules containing heteroatoms and to produce a complex with aromatic components. Most often used are the Cu^{2+} and Zn^{2+} (ref. 76). Cu^{2+} has been used to purify an *Aspergillus niger* carboxypeptidase[77,78].

Purification on heparin, classically used for the proteases involved in blood clotting, should also be mentioned.

Numerous experiments have been carried out to try to improve the yield of affinity purifications and to adapt them to processes able to deal rapidly with large volumes. Some newer strategies are outlined below.

3.1.1.5.5. Counter-flow affinity filtration. This method combines the specificity

TABLE 1

AFFINITY CHROMATOGRAPHIC TECHNIQUES APPLIED TO PROTEOLYTIC ENZYMES

DFP = Difluorophosphate.

No.	*Enzyme*	*Ligand*	*Eluent*	*Ref.*
1	Acid protease	Pepstatine	Urea	86, 87
2	Amino peptidase	Leu–Gly	NaCl, Zn	88–90
3	Asparaginase	L-Asp	NaCl, Asp	91
4	Carboxypeptidase A	Phenyl propionates, *p*-aminobenzyl succinate	KCl	92 93
5	Carboxypeptidase B	D-Arg Protease inhibitor	NaCl, pH	94
6	Carboxypeptidase N	Aminobenzoylarginine	Guanidoethylmercapto succinate	95
7	Carboxypeptidase	Potato inhibitor	pH	58
8	*Aspergillus niger* carboxy-peptidase	Cu^{2+}–IDA Cu^{2+}–tyrosine	Acetate + EDTA	96
9	Cathepsin	Pepstatin Con-A	NaCl pH	87 97
10	Chymotrypsin	CBZ–(ω-NO_2)–Arg D-Tryptophan methyl ester	Phosphate buffer Acetic acid	37
11	Mammalian collagenase	Heparin, Zn^{2+}	Cacodylate buffer + NaCl Sodium cacodylate buffer, pH	98
12	Human collagenase	Collagen, Arg	NaCl	39,99
13	Porcine collagenase	Zn^{2+}	pH + EDTA	98
14	Dipeptidyl-peptidase	4-Phenylbutyl–Gly–Pro	Ethylene glycol, NaCl	100
15	Elastase	Elastin, $(Ala)_3$	Salt, Ala	99,101,102
16	Tissue factor apoprotein	Con-A	α-Methyl D-glucoside	103
17	Bovine factor VII	Benzamidine	Guanidine, HCl	104
18	Human factor XII (Hageman factor)	Lysine	$(NH_4)_2SO_4$	105
19	Bovine factor XI (plasma thromboplastin antecedent)	Heparin Benzamidine	NaCl + DFP Imidazole, HCl + guanidine, HCl + NaCl	106
20	Bovine factor X (Stuart factor)	Benzamidine	Guanidine, HCl	107
21	Formylmethionine-aminopeptidase	N-Formylbestatine	NaCl	108
22	Human prekallikrein	Heparin Benzamidine Con-A Agmatine	NaCl Guanidine, HCl α-Methyl D-glucoside NaCl	109, 110
23	Porcine kallikrein	Aprotinine	pH	111
24	Human kallikrein	Aprotinine Aminobenzamidine	Phosphate buffer Benzamidine, HCl	109, 110
25	Acid protease	Pepstatine	Urea gradient	87, 112
26	Neutral protease	N-phenylphosphenyl–Phe–Phe	pH	113
27	Pepsin	Poly(L-Lysine) ε-Aminocaproyl–L-Phe–D-Phe–OCH_3	NaCl NaCl	114, 115
28	Plasmin	Lysine	pH + NaCl	116
29	Post-proline hydrolase	CBZ–Pro–D-Ala	CBZ–Pro–Phe	117

TABLE I (*continued*)

No.	*Enzyme*	*Ligand*	*Eluent*	*Ref.*
30	Proteinase	4-Phenylbutylamine	NaCl, urea	118
		Soybean trypsin inhibitor D-Tryptophan methyl ester	$CaCl_2$	119, 120
31	*Aspergillus* proteinase	ε-Aminocaproyl–Phe–D-Phe–OCH_3	NaCl	121
32	Renin	Pepstatine,	pH	122, 123
		haemoglobin octapeptide (D-Leu[6])	pH Sodium acetate	124
33	*S. aureus* staphylocoagulase	Bovine prothrombin	NaSCN	125
34	Staphylokinase	DIP-canine-plasmin	pH	126
35	Subtilisin	*p*-(ω-aminoethyl)phenyl boronic acid	pH + glycerol	127
36	Thrombin	Aminobenzamidine	Benzamidine	128
37	Trypsin	Benzamidine	pH	
		CBZ–(ω-NO_2)–L-Arg	pH + semicarbazide	5
		Soybean trypsin inhibitor	pH + benzamidine	41
38	Tyrosinase	Con-A	α-Methyl D-mannoside	129

of affinity techniques with the efficiency of membrane separation. The substance to be purified, or the impurities if they are not too numerous and of a known nature, is specifically adsorbed on a solid support or an insoluble polymer. The molecular mixture containing the adsorbed substance is separated from the contaminants by filtration. The enzyme is desorbed by a suitable polymer, then the support and polymer are recycled. Such a process has developed for continuous use at the outlet of a bioreactor and has been tested on various biological molecules such as Con A and alcohol dehydrogenase[79,80], but has not, as far as we are aware, been used for proteolytic enzymes.

3.1.1.5.6. Affinity partitioning. The main inconvenience of the previous process is the few affinity sites that are available and the difficult access to such sites. To overcome this disadvantage, some workers have tried to avoid the difficulties by using a water soluble polymeric matrix on which the chosen ligand was coupled. Adamski-Medda *et al.*[81] thus separated trypsin from chymotrypsin by using *p*-aminobenzamidine bound to dextran. Choe *et al.*[82] separated the same enzymes using soybean trypsin inhibitor. Disappointing results ensued, with purification yields of 65% in the former instance and 81% in the latter, and the recovery rate of about 55% shows the difficulties of finding a macroligand specific enough for the enzyme to be purified. Some workers have avoided the difficulty by using synthetic macroligands. Luong *et al.*[83] synthesized an acrylamide polymer upon which *m*-aminobenzamidine groups are coupled. The capacity of such a ligand is close to the theoretical value[81]. By eluting with L-arginine, it is possible to recover 90% of the starting trypsin with a purity of 98%. Such a system can be operated continuously.

A number of other ligand polymers useful for affinity partitioning and methods for their preparation have been summarized elsewhere[84,85]. The amount of protein that can be included is limited by the solubility in the two phases, but 50 g of protein per

kilogram of partition system can often be used[6]. This technical approach is very promising but its industrial application is limited owing to the cost.

Affinity chromatographic techniques that have been applied to proteolytic enzymes are surveyed in Table 1.

3.1.2. High-performance liquid chromatography (HPLC)

The high-resolution HPLC processes are widely used in analysis and research and, because of their speed and efficiency, they are beginning to be used on a preparative scale. In order to limit costs and maximize production in preparative chromatography, the column should be overloaded. Ghodbane and Guiochon[130] described the prediction of the optimum extent of overloading in relation to the required recovery and degree of purity of the final products.

The most commonly used techniques are high-performance size-exclusion chromatography and high-performance ion-exchange chromatography.

3.1.2.1. High-performance size-exclusion chromatography (HPSEC)

HPSEC enables eluted molecules to be separated according to their decreasing molecular weight and size. The breakthrough with this technique was associated with the development of highly resistant columns operating at high back-pressures, *i.e.*, with hydrophilic polymer gels, diol-bonded silica, hydrophilic grafted or coated silica gels and cellulose derivatives[131,132]. HPSEC is applied in four areas: prefractionation, analytical separations, molecular weight determinations and preparative isolation. It is also suitable in the monitoring of the time course of enzymic reactions[132]. It has several advantages: proteins are eluted rapidly with relatively narrow peaks; as an isocratic system generally suffices, it is cheap and makes it possible to isolate easily a few milligrams of enzyme[133]. This compensates for the disadvantages involved, such as limited peak capacity and the fact that it is never entirely quantitative because of parasitic adsorption phenomena between the enzyme and the matrix[134]. The resolution depends on the column (mass loadability, fractionation range), flow-rate and size and shape of the proteins[135]. In order to increase the resolution, several column of decreasing fractionation range can be used. It is strongly recommended to collect only the centre of the peaks and recycle the remainder. The flow-rates vary between 0.5 and 1.5 ml/min in the analytical and between 8 and 10 ml/min in the preparative mode. The commonest commercial columns are those from Toyo Soda (SW and TSK series). Merck (LiChrosorb Diol and LiChrospher), Waters Assoc. (Protein-Pak series), Synchrom (Synchropak) and Lachema (Separon).

The main proteases thus purified are trypsin[133], chymotrypsin[133], pepsin[134], numerous kinases[136] and a gelatinase[135].

3.1.2.2. High-performance ion-exchange chromatography (HPIEC)

In the HPIEC mode, the enzymes are separated not only according to their charge but also their hydrophobicity and molecular weight, The effects of the mobile phase, of ionization and a salt slope play a major role in separations[137–140]. The usual gradient slope is a 3.3% salt gradient accross the column. Mobile phase velocities of 7.2 ml/min per cm^2 cross-sectional area (1 ml/min in a 42–50 mm column) are average[137]. Operating at this velocity with a 3–6%/min gradient slope will give good separations in 15–30 min. Halving the mobile phase flow-rate has been shown to increase the

resolution 1.5-fold when a 1.66%/min gradient slope was used. In this instance, the total separation time would be 60 min. The two variables that have been found to be most useful in controlling retention and selectivity are the pH of the mobile phase and the nature of displacing salt. The influence of the major ions on the retention and resolution of various proteins has been studied by Regnier[137]. The main columns in use are the Mono S and Mono Q (Pharmacia), the Synchropak AX, CM and QX series (Synchrom) and the IEX series (Toyo Soda). In order to separate enzymes with closely similar p*I* values, chromatofocusing (Mono P columns, Pharmacia) is occasionally used[141,142].

3.1.2.3. Reversed-phase high-performance liquid chromatography (RP-HPLC)

This very selective method has the inconvenience of being enzyme denaturing. A number of trials were carried out in order to find supports and elution conditions that permit biological activity to be retained[143–145] by adding ion-pairing agents[146] and stabilizers. Utilization of volatile organic acids (formic, acetic, trifluoroacetic and heptafluoroacetic acids) and of calcium chloride made it possible to recover biological activity after elution by rapid evaporation of the organic solvent under nitrogen[147,148], or by modifying the pH buffer or molarity just after separation[143]. In order to increase the recovery of proteins of MW > 30 000 daltons, some workers advise the use of "end-capped" short linked phases, RP-18 or RP-4, especially in the semi-preparative mode[149–151]. Optimizing the column and separation by sample pretreatment and altering the mobile phase conditions have been extensively studied[152,153]. However, this technique is mainly used for low-molecular-weight enzymes. Because of risks of denaturation, it is advisable to use this technique for checking the purity of a given sample or for purifying small amounts of product for structural investigations.

3.1.2.4. High-performance hydrophobic interaction chromatography (HPHIC)

HPHIC with microparticulate supports, introduced in 1983[154], made it possible to separate proteins rapidly with high resolution and to recover almost quantitatively the injected sample with good preservation of the activity[155]. The supports used contain a silica matrix[156–161] or hydrophylic polymers[162–164]. The hydrophobicity of the support is adjusted so that the proteins can be adsorbed in ionic, anti-chaotropic solutions within the molarity range 1–2 M. The selectivity was shown to change with eluent pH[165]. Gradients often used are ammonium sulphate or potassium phosphate with molarity varying between 1.5–2.5 and 0.05–0 *M* over a 30–60-min period. The flow-rates usually used are 0.5–1 ml/min. Kato[166] reported a detailed study on the various parameters that can influence separation: hydrophobicity of the support ligand, column length, initial salt concentration, type of salt and slope of salt concentration, elution curve, pH, flow-rate, temperature, addition of organic solvents, chaotropic agents or surfactants and sample loading.

3.1.2.5. High-performance affinity chromatography (HPAC)

HPAC combines the high specificity of affinity techniques with the efficiency, sensitivity and speed of HPLC techniques. For proteolytic enzymes, the ligands used may be dyes (Procion Yellow, Cibacron Blue F3G), proteins (soybean trypsin inhibitor, Con-A), antibodies, metals or various other substances. Fassina and

Chaiken[167], Larsson[168] and Olsson[169] have studied the various techniques used for coupling of ligands, to control the determination of bound ligands and to optimize separations. It should be noted that a commercial column (Ultraffinity, Beckman) is available on which the user can couple a larger number of ligands. This technique is very interesting for analytical work but has been little used with proteolytic enzymes.

Proteases separated by this technique include hexokinase (Procion Green MX-5BR)[170], carboxypeptidases (Procion Yellow H-A)[170], various proteolytic enzymes of the complement system C1r, C1s, C2a, etc. (antibodies)[171], chymotrypsin (soybean trypsin inhibitor)[168] and pepsin (L-Phe–D-Phe–OCH_3, methacrylate support)[172].

Examples of HPLC techniques applied to the purification of proteolytic enzymes are summarized in Table 2.

TABLE 2

EXAMPLES OF HPLC TECHNIQUES APPLIED TO THE PURIFICATION OF PROTEOLYTIC ENZYMES

Technique	*Column*[a]	*Ref.*
HPSEC:		
A. melleus semi-alkaline protease	TSK G 3000 SW (Tosoh)	173
Pepsin	TSK G 3000 SW (Tosoh)	174
Leucine aminopeptidase, trypsin, pepsin, α-chymotrypsin	LiChrosorb Diol (Merck)	175
Mastocyta tryptase	TSK G 3000 SW (Tosoh)	176
HPIEC:		
Saccharomyces cerevisiae protease	Mono Q (Pharmacia)	177
Muscular cysteine proteinase	Mono Q (Pharmacia)	178
Entamoeba histolytica proteinase	Mono P (Pharmacia)	179
Human metalloendoprotease	Mono Q (Pharmacia)	180
E. coli membrane protease	Mono P (Pharmacia)	181
Rat thiol proteases	Mono P (Pharmacia)	182
Proteases (T cells)	Mono Q (Pharmacia)	3
Chymopapain	Mono S (Pharmacia)	183
A. niger semi-alkaline protease	Mono Q (Pharmacia)	184
Bovine adrenomedullary granules protease	Mono Q (Pharmacia)	185
HPLAC:		
Trypsin	STI–LiChrospher 500 NH_2 (Merck)	186
A. niger protease–pepsin	ω-Aminocaproyl-L-Phe-D-Phe-OCH_3 Separon H 100[b]	172
RP-HPLC:		
Trypsin	μBondapak C_{18} (Waters Assoc.)	187
Papain	LiChrospher 500-*n*-butyl (Merck)	33
RP-HIC:		
Human metalloendoprotease	TSK-phenyl 5 PW (BioRad Labs.)	180
Procarboxypeptidases A and B	TSK-phenyl 5 PW (BioRad Labs.)	188
Proelastase	TSK-phenyl 5 PW (BioRad Labs.)	188
Renin	TSK-phenyl 5 PW (Toyo Soda)	189

[a] STI = Soybean trypsin inhibitor.

[b] Column packed by workers.

3.2. Electrophoretic techniques

3.2.1. Preparative isoelectric focusing

Isoelectric focusing is a very attractive method for the fractionation and isolation of enzymes. It permits a fairly large amount of product to be purified with excellent resolution and load capacity. The method concentrates and separates molecules different each other in only 10^{-2} pH unit[190]. Four kinds of matrices are used: polyacrylamide gels, agarose, granulated gels (Sephadex G-200, Sephacel, Bio-Gel 60) or rehydratable gels, the choice depending on the amount and to the molecular weight of the enzymes to be separated. Laboratory-scale techniques on polyacrylamide or Sepharose gels enable fractions up to 100–150 mg and large-scale fractionation permits several grams to be purified in one step by using density gradient columns and layers of granulated gels[191,192]. Resolution is influenced by the field strength and the shallowness of the pH gradient. Once focusing is finished, the enzymes must be located in the gel layer and separated at low temperature by rapid, simple and preferably non-destructive methods. Speed is important because keeping the gels without a voltage or at a reduced voltage can lead to broadening of zones by protein diffusion[191]. The recovery depends on a number of factors, such a proper separation including elution from the gel, additional steps that may be necessary for removal of ampholytes and/or concentration of the isolated fraction. Many techniques, such dialysis, electrodialysis, ultrafiltration, salting-out, gel chromatography, ion-exchange or hydrophobic chromatography, two-phases extraction with *n*-pentanol and, more recently, electroosmosis have been suggested for the removal of ampholytes[188]. An alternative is "Trans elution" which consists of the sideways transfer of the macromolecules under the influence of an electric field from the gel slab[189]. The electro-eluted proteins remain in solution and are easily recovered by spin-drying the support. It may be possible to recover 85–92% of the crude sample[190]. The recovery also depends on the load capacity; loads from 0.5 to 10 mg/ml gel increase the recovery of Pronase from 14 to 80%[190].

These techniques are mostly used in research and to separate rapidly analytical samples. The various methodologies have been detailed by various workers[190,192,193].

3.3. Particular instances

3.3.1. Metal-free enzymes

It is sometimes necessary to utilize metal-free enzymes, for instance to study enzyme–metal interactions or to keep an enzyme in its crystalline stage (carboxypeptidase A)[194]. Suitable apoenzymes are prepared by using metal-chelating agents. As proteases are often zinc-activated metalloenzymes, 1,10-phenanthroline is used[195]. Proteases purified in this way include *Acromonas* aminopeptidase[196], angiotensin-converting enzyme[197], *Bacillus subtilis* neutral protease[198], carboxypeptidase A[199,200] and B[201], a bovine aminopeptidase[202], procarboxypeptidase A[202], *Streptomyces griseus* carboxypeptidase[204] and thermolysin[205].

4. ELUATE CONCENTRATION

Following chromatography, the media must be concentrated. The most widespread methods are ultrafiltration and dialysis. Freeze-drying is sometimes used.

4.1. Concentration by ultrafiltration

This is the most common technique. Diffusive ultrafiltration, in which molecules are removed by molecular diffusion under the action of a concentration or activity gradient, is regularly used. To reduce the phenomena of concentration polarization and enzymic adsorption, tangential filtration with a polysulphone or cellulose nitrate membrane is mostly used. The main application have been discussed by Kusiak *et al.*[206]. It is also possible to use diafiltration; such a method of washing with a regulated volume is suitable for eliminating mineral salts and low-molecular-weight substances.

4.2. Concentration by freeze-drying

Although freeze-drying is employed classically for the concentration of enzymes, its use is limited by the fact that unless the salt concentration of solutions is sufficiently reduced, eutectic mixtures may be formed. This may lead to incomplete drying or severe foaming and enzyme denaturation[1]. To avoid denaturation and loss of activity, it is essential to operate under very strict pH conditions, which vary according to the enzymes, and to suppress or limit the final heating during deadsorption.

The "drop dialysis method", developed by Marusyk and Sergeant[207] for dialyse[129] small-volume samples of DNA is interesting. This technique, initially applied to DNA, makes it possible to prepare quickly small samples for electrophoresis or any other analytical method, with high recoveries and a tolerable loss of enzyme activity[208].

5. ENZYME CRYSTALLIZATION

The operating conditions vary according to the enzymes. It is nevertheless necessary to control certain factors, such as pH, temperature and precipitants. There are three distinct phases to be considered: nucleation, post-nucleation and cessation of growth[209]. Crystal formation is possible only at the critical point of supersaturation in an appropriate environment (ionic strength, pH, temperature). Crystals continue to grow as long as the state of supersaturation exists. The optimum protein concentration is *ca.* 10–20 mg/ml. It is essential to add the precipitating agent in small amounts and to follow nucleation[210]. One most often operates at the pH of activity (*i.e.*, pH of maximum enzyme activity), at room temperature or in a cold chamber (4–6°C). Gilliand and Davies[209] published a review that summarizes the chief precipitating agents and the different techniques in use, the most widespread being ammonium sulphate, PEG and 2-methyl-2,4-pentanediol.

6. ENZYME PURIFICATION MONITORING

Enzyme purification is most often carried out by electrophoresis and sometimes HPLC (RP-HPLC, HPSEC or HPIEC). UV detection at 280, 254 or 214 nm provides an excellent means of controlling product purity and quantifying yields. Electrophoretic techniques currently used are analytical electrophoresis, sodium dodecyl sulphate (SDS) electrophoresis and electrofocusing; immunoelectrophoresis is also used. Burton *et al.*[152] explored a new strategy which couples RP-HPLC with SDS electrophoresis.

6.1. Techniques for enzyme separation

6.1.1. Analytical electrophoresis

When it is necessary to separate intact proteins by a non-destructive means for later assessment of biological activity, gels must be prepared under non-denaturing conditions, *i.e.*, polyacrylamide gel electrophoresis (PAGE)[211,212]. As the speed of electrophoretic migration of a protein depends not only on its charge but also on its molecular size and shape, it is possible to separate two enzymes with an identical net charge but of different size by varying the pore size of the support as a function of the acrylamide (range about 2.5–30%, w/v) and the amount of cross-linker used. Gels with less than 2.5% permit proteins with a molecular weight close to 10^6 daltons to be separated and gels with 30% do the same for polypeptides with molecular weights close to 2000 daltons[213]. Separation by charge is achieved by operating between pH 3 and 11 to allow for maximum charge differences between neighbouring protein species[214].

To analyse very complex media or isolate enzymes in very small amounts, it is possible to increase the resolution by two-dimensional operation. Since O'Farrel's original publication[215], numerous modifications have been introduced to improve reproducibility[216–219] and increase the resolution and detection of proteins[220]. The use of a heating apparatus, of denaturing agents such as β-mercaptoethanol[218,221,222], dithioerythritol (DTE) or dithiothreitol (DTT)[216,218] and of zwitterionic detergents[218] has been proposed. Simplification and improvement of the O'Farrel method made it possible to obtain a better separation and to increase the detection sensitivity by reducing the background noise[223].

For analytical purposes, media are often separated by denaturing electrophoresis, the most often used being the SDS techniques of Laemmli[224] or Neville[225]. The advantage of Neville's system is that the lengthy washing required to remove glycine from gels when Tris–glycine is used is avoided. Treatment with denaturing agents. SDS (1–2%), with or without reducing agents, dithiothreitol (40–100 m*M*) or 2-mercaptoethanol (up to 1 *M*) reduces disulphide bonds. The only separative criterion is protein size.

It is possible to prepare gels with an increasing acrylamide concentration gradient (generally 5–20%), which has two advantages, first for separating a larger range of molecular weights and second for increasing the resolution. Electrophoresis can be performed with a single buffer (continuous electrophoresis) or with several buffers (discontinuous electrophoresis). The latter technique permits proteins to be concentrated with extremely small zones prior to their migration. Specific details of the procedure have been described by Blackshear[214]. A single band in SDS electrophoresis is usually accepted as a criterion of purity.

6.1.2. Isoelectric focusing

Isoelectric focusing offers two distinct advantages over the usual methods of electrophoresis: it gives a high resolution, proteins differing in p*I* by 10^{-3} to 10^{-2} being resolved, and it is independent of time so deterioration of the zone definition with time is almost negligible. The resolution is improved by using high field strengths (100–300 V/cm) in thin (0.2–1 mm) gels layers.

Affinity electrophoresis and isotachophoresis can also be used. Kolodzeiskaia[226] recently published a paper in which electrophoretic techniques applied to proteolytic enzymes were reviewed.

6.2. Enzyme localization in gels

6.2.1. Non-specific detection

Such techniques imply an irreversible alteration of the enzyme and their only interest is to permit its observation. Coomassie R_{250}[227] and G_{250} Blue[228] and silver staining[229] are commonly used. The sensitivities are about 50 ng and 1 ng per spot, respectively. Because numerous proteases are glycoproteins, it is of interest to detect them more specifically. Three methods are used[230], the thymol–sulphuric acid method, the periodic acid–Schiff base method and the fluorescein isothiocyanate–labelled lectin method.

6.2.2. Specific detection

It is often relevant to preserve enzymic activity and to determine it. There it is necessary to eliminate the electrophoretic reagents that are likely to interfere in the determination (ampholine, SDS, etc.) and sometimes to renature the enzymes. Different techniques are used, such as removal of SDS by incubation in buffered 25% isopropanol or renaturing of enzyme by using a combination of non-ionic detergent, glycerol and substrate; subsequently several approaches can be chosen[231].

6.2.2.1. Elution of enzymes from the gel

The gel is cut into pieces and the enzymes are eluted with an appropriate buffer and assayed by conventional methods. The measured activity depends on the electrophoretic technique used (more or less denaturing) and of the recovery from the gel. Spectrophotometric assays are widely used to detect proteolytic enzymes. These use fluorochrome[232–236] or chromogenic indicators conjugated to the substrate[237–241]. Soluble substrates[241,244] require the removal of undegraded material (usually trichloroacetic acid precipitation), unless they have been attached to a solid support, such as Sepharose[236,238]. Insoluble substrates such as azocoll[240] can be removed by centrifugation. Proteolytic activity is determined by measuring the amount of indicator released in the supernatant. Alternatively, indicators such as fluorescamine[234] or *o*-phthaldehyde [241] may be added to the supernatant to detect digestion products. These assays can detect trypsin, collagenase or elastase[234,235] and chymotrypsin[233] at concentrations of 50 and 1 mg/ml, respectively. An alternative is to used a radiolabelled substrate such as [^{14}C]collagen[242,243], [^{3}H]elastin[244,245] or [^{125}I]fibrinolectin[246] and casein[247]. All of these assays rely on the release of radiolabelled products into the supernatant, where they are determined by gamma or scintillation counting. An assay using radioiodine–gelatin as substrate was published recently[248]. This assay is able to detect elastase, trypsin and collagenase at concentrations of 1 ng/ml or less.

6.2.2.2. Staining for enzymic activity in situ after electrophoretic detection

Heeb and Gabriel[231] have reported five groups of techniques:

(i) simultaneous capture, where the substrate is converted by the enzyme into a product that couples immediately with a reagent present in the incubation mixture to form an insoluble coloured product;

(ii) post-incubation coupling, in which incubation of substrate with enzyme results in a product, then a reagent is added to the medium to yield coloured material;

(iii) autochromic methods, which make possible direct observation of enzymic activity by changes in the optical properties of either the substrate or reaction product;

(iv) sandwich-type incubation, which uses a matrix containing an auxiliary indicator enzyme or high-molecular-weight substrates; incubation of the separating gel with the matrix permits localization of enzymic activity; and

(v) copolymerization of substrate in the gel, which is used for high-molecular-weight substrates such as gelatin or casein. It is often necessary to prevent the enzyme from acting on the substrate during electrophoresis under enzymic conditions, *e.g.*, inclusion of an inhibitor or chelating agent, use of a less than optimal pH, or other means. Conditions of optimum activity can be restored during the subsequent incubation period in order to detect enzyme activity.

The main techniques used for localizing proteases *in situ* are reported in Table 3.

TABLE 3

LOCALIZATION OF ENZYME ACTIVITY

No.	*Enzyme*	*Substrate*[a]	*Detection*[b]	*Ref.*
1	Acrosin (E.C. 3.4.21.10)	Arg–β-naphthylamine	Coupling with Fast Black	249
2	Aminopeptidase (E.C. 3.4.11.4)	L-Leu–Gly–Gly	Coupling with amino acid oxidase, PMS	231, 250
3	Carboxypeptidase (E.C. 3.4.17)	Carbonaphthoxy–Phe	Coupling with Diazo Blue B	251
4	Cathepsin B (E.C. 3.4.22.1)	Z Arg–Arg–methoxynaphthyl-amide	Coupling with Fast Garnet, GBC/scan	252
		Azocoll	TCA precipitation	252
5	Chymotrypsin (E.C. 3.4.21.1)	Z Tyr–*p*-nitroanilide	Diazotization of *p*-nitroanilide	254
6	Dipeptidase (E.C. 3.4.14)	L-Leu–Leu	Coupling with amino acid oxidase, PMS	231, 250
7	Elastase (E.C. 3.4.31.11)	N-Acetyl-DL-Ala α-naphthyl ester	Coupling with Fast Black	231, 255, 256
		Elastin–orcein	Scan at 550 nm	
			Clear zones	
8	Proteinases (E.C. 3.4)	Casein, azocoll, gelatin	TCA precipitation, scan at 520 nm	257, 258
		Fluorescein–gelatin	Salt precipitation, fluorescence	259–261
		FITC–casein	Clear zones	262, 263
9	*Aspergillus* proteinases (E.C. 3.4)	Azocasein	Clear zones	264
10	Thermolysine (E.C. 3.4.24.4)	Cytochrome *c*	TCA precipitation	253
11	Trypsine (E.C. 3.4.21.4)	Azocoll	TCA precipitation + scan	253, 265, 266
		BAPNA	Diazotization of *p*-nitroanilide	256
		Benzoyl–Arg–Arg *p*-nitroanilide		
12	Subtilisin (E.C. 3.4.21.14)	Benzoxyl–Gly–Gly Leu–*p*-nitroanilide	Product visible under UV light	267
13	Urokinase	Plasminogen, fibrin	Clear zones	268
	Plasminogen activators (E.C. 3.4.31.31)	Plasminogen, gelatin	Amido Black staining	

[a] Z = Carbobenzoxy; FITC = isothiocyanate–fluorescein; BAPNA = benzoylarginine-*p*-nitroanilide.

[b] PMS = Phenazine methosulphate; TCA = trichloroacetic acid; INT = iodophenyl nitrophenyl tetrazolium; Fast Garnet GBC base = 4-amino-2',3-dimethylazobenzene.

6.2.3. Western blotting

In order to facilitate the detection of an enzyme in a very complex medium (*e.g.*, biological extract, fermentation juice) it may be interesting to try the western blot technique. After the enzyme has been separated by a suitable electrophoretic technique and renaturation, it is transferred on to a membrane, then detected by incubation in the presence of an antibody or a specific ligand and rendered visible. There are seven practical stages of operation:

(i) separation of the crude extract by electrophoresis;

(ii) washing away the gel before transfer and renaturation by removal of detergents:

(iii) transfer (usually electrotransfer); the transfer conditions, *i.e.*, pH, ionic strength, presence of methanol, are determined by the nature of the required protein and the commonly used membranes are made of nylon, nitrocellulose or poly(vinylidene dichloride)[269];

(iv) pre-hybridization, *i.e.*, eliminating non-specific support fixation sites which account for background noise;

(v) detection with a non-specific dye (Coomassie Blue, Amido Black, aurodye), by marking with a radioactive isotope (^{32}P, ^{125}I)[270], a fluorescent marker, an antibody avidin–biotin system[271] or immuno-gold–silver staining[272]; zinc-binding enzymes can be probed with radioactive zinc (^{65}Zn) and detected by autoradiography[273];

(vi) elimination of free ligand by washing; and

(vii) signal spotting through coloration, fluorescence or autoradiography.

The practical aspects of such techniques have been discussed by Durand[274] and Walker[275]. This very sensitive method, the detection threshold of which can be close to 1 ng/mm^2, is very interesting for the detection of therapeutically significant enzymes, *e.g.*, collagenase in some forms of cancer[276]. It may also possible to detect specifically active enzymes by performing the coupling reaction after transfer on to a membrane on which have been previously fixed auxiliary enzymes specific for the reactive product[276].

7. ACKNOWLEDGEMENT

The author is indebted to Prof. H. Pourrat for useful comments.

8. SUMMARY

A purification scheme has been established and three major steps defined: extraction and solubilization of the enzyme in the medium, obtaining the crude enzyme and selective purification. For each step, the applicable techniques, with their advantages and incoveniences, and the classical parameters in use are discussed. Affinity liquid chromatography and high-performance liquid chromatographic processes have been particularly developed. Techniques for enzyme crystallization and methods for monitoring enzyme purification are considered.

REFERENCES

1 J. Helling and B. W. Phillips, in A. Wiseman (Editor), *Handbook of Enzyme Biotechnology*, Ellis Horwood, Chichester, 1975, p. 59.
2 H. Hustedt, K. H. Kroner, H. Schütte, U. Menge and N. Papamichael, *Bioengineering*, 1 (1986) 12.
3 G. Johansson, *Methods Enzymol.*, 104 (1984) 356.
4 G. Johansson, in H. Welter, D. E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems*, Academic Press, Orlando, 1985, p. 161.
5 T. B. Choe, P. Masse and A. Verdier, *Biotechnol. Lett.*, 8 (1986) 163.
6 S. J. Stocks and D. E. Brooks, *Anal. Biochem.*, 173 (1988) 86.
7 P. A. Albertsson and G. Birkenmeier, *Anal. Biochem.*, 175 (1988) 154.
8 H. Hustedt, K. H. Kroner, U. Menge and M. R. Kula, *Trends Biotechnol.*, 3 (1985) 139.
9 K. H. Kroner, H. Hustedt, S. Granda and M. R. Kula, *Biotechnol. Bioeng.*, 20 (1978) 1967.
10 M. R. Kula, *Ann. N.Y. Acad. Sci.*, 341 (1981) 354.
11 H. Hustedt, B. Börner, K. H. Kroner and N. Papamichael, *Biotechnol. Techn.*, 1 (1987) 49.
12 K. C. Ingham, *Methods Enzymol.*, 104 (1984) 351.
13 T. F. Bussy and K. C. Ingham, *J. Biochem. Biophys. Methods*, 2 (1980) 191.
14 K. C. Ingham and T. F. Bussy, *Chem. Eng. Commun.*, 7 (1980) 315.
15 D. A. Scott, *Biochem. J.*, 28 (1934) 1592.
16 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1922) 384.
17 E. J. Cohn, F. R. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. M. Mittlerman, R. F. Mouton, K. Schmid and E. Uroma, *J. Am. Chem. Soc.*, 72 (1950) 465.
18 P. A. Roche and S. V. Pizzo, *Arch. Biochem. Biophys.*, 256 (1987) 265.
19 V. C. W. Tsang, J. M. Peralta and A. R. Simons, *Methods Enzymol.*, 92 (1983) 377.
20 E. L. Giroux and R. I. Henkin, *Biochim. Biophys. Acta*, 273 (1972) 64.
21 P. G. Zaworski and G. S. Gil, *Anal. Biochem.*, 173 (1988) 440.
22 J. Schneider, J. M. Harris and M. Yalpani, in J. M. Harris and M. Yalpani (Editors), *Partitioning in Aqueous Two-Phase Systems*, Academic Press, Orlando, FL, 1986, p. 126.
23 J. L. Tayot, *Biofutur*, 51 (1986) 69.
24 G. Segal, *Biofutur Technoscope*, No. 12, July (1987) 5.
25 L. A. Æ. Sluyterman and J. Wijdenes, in B. J. Radola and D. Graesslin (Editors), *Proceedings of International Symposium Electrofocusing and Isotachophoresis*, Walter de Gruyter, Berlin, 1977, p. 463.
26 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31.
27 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 429, 441.
28 M. T. Martin, *Methods Enzymol.*, 158 (1988) 15.
29 S. Shaltiel, *Methods Enzymol.*, 104 (1984) 69.
30 T. A. Stewart, J. A. Weare and E. G. Erdös, *Methods Enzymol.*, 80 (1981) 450.
31 T. C. Carilli, L. Cameron Wallace, L. M. Smith, M. A. Wong and J. A. Lewicki, *J. Chromatogr.*, 444 (1988) 203.
32 R. Zwilling and H. Neurath, *Methods Enzymol.*, 80 (1981) 633.
33 G. Bernardi, *Methods Enzymol.*, 22 (1971) 325.
34 H. Hirano, T. Nishimura and T. Iwamura, *Anal. Biochem.*, 150 (1985) 228.
35 R. K. Scopes, *Anal. Biochem.*, 165 (1987) 235.
36 S. I. Ishii and K. I. Kasai, *Methods Enzymol.*, 80 (1981) 842.
37 T. V. Wilkinson, A. H. Patel and R. M. Schultz, *Sep. Sci.*, 11 (1976) 385.
38 C. Kattner and E. Shaw, *Anal. Biochem.*, 80 (1981) 826.
39 I. Emöd and B. Keil, *FEBS Lett.*, 77 (1977) 51.
40 N. Sakuragawa, K. Takahashi and T. Ashizawa, *Acta Med. Biol.*, 25 (1977) 119.
41 A. H. Patel and R. M. Schulz, *Biochim. Biophys. Res. Commun.*, 104 (1982) 181.
42 D. L. R. Hwang, K. T. D. Lin, W. K. Yang and D. E. Foard, *Biochim. Biophys. Acta*, 495 (1977) 369.
43 D. E. Foard and K. D. Lin, *Abstracts, Euroanalysis III*, Abstract 27, August 1978.
44 W. M. Roschlan, *Folia Haematol.*, 101 (1974) 22.
45 H. Fritz, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 893.
46 K. D. Lin, D. L. Hwang and D. E. Foard, *J. Chromatogr.*, 195 (1980) 385.
47 A. Kanamori, N. Seno and I. Matsumoto, *J. Chromatogr.*, 363 (1986) 231.
48 G. Schmer, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 810.

49 L. Holmberg, B. Bladh and B. Åstedt, *Biochim. Biophys. Acta*, 445 (1976) 215.
50 R. L. Heimark and E. W. Davie, *Biochemistry*, 18 (1979) 5743.
51 K. Tasaka, *Pharmacology*, 21 (1980) 43.
52 C. Sampaio, S. C. Wong and W. Shaw, *Arch. Biochem. Biophys.*, 165 (1974) 133.
53 D. M. Monroe, G. B. Sherril and H. R. Roberts, *Anal. Biochem.*, 172 (1988) 427.
54 V. K. Akparov and V. M. Stepanov, *J. Chromatogr.*, 155 (1978) 329.
55 E. Millquist, H. Petersson and M. Ranby, *Anal. Biochem.*, 170 (1988) 289.
56 J. Pohl, M. Zaoral, A. Jindra, Jr. and V. Kostka, *Anal. Biochem.*, 139 (1984) 265.
57 P. Rauber, P. Wikstrom and E. Shaw, *Anal. Biochem.*, 168 (1988) 259.
58 A. J. Barret, *Methods Enzymol.*, 80 (1981) 771.
59 G. M. Hass and C. A. Ryan, *Methods Enzymol.*, 80 (1981) 778.
60 M. T. Everitt and H. Neurath, *FEBS Lett.*, 110 (1980) 292.
61 C. R. Lowe and J. C. Pearson, *Methods Enzymol.*, 104 (1984) 113.
62 Y. D. Clovis, M. J. Goldfinch and C. R. Lowe, *Biochem. J.*, 197 (1981) 203.
63 P. Hughes, C. R. Lowe and R. I. Sherwood, *Biochim. Biophys. Acta*, 700 (1982) 90.
64 G. Segal, *Biofutur Technoscope*, No. 14, October (1987) 18.
65 A. Kamra and M. Gupta, *Anal. Biochem.*, 164 (1987) 405.
66 R. Lotan and G. L. Nicolson, *Biochim. Biophys. Acta*, 559 (1979) 329.
67 R. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
68 J. J. Lanzillo, R. P. Cynkin and B. L. Fanburg, *Anal. Biochem.*, 103 (1980) 400.
69 Y. Mori, *J. Chromatogr.*, 189 (1980) 438.
70 R. A. Wetsel, M. A. Jones and W. P. Kolb, *J. Immunol. Methods*, 35 (1980) 319.
71 R. B. Sim, *Methods Enzymol.*, 80 (1981) 26.
72 J. L. Lane, H. Ekert and A. Vafiadis, *Thromb. Haemostasis*, 42 (1979) 1306.
73 J. A. Katzmann, D. Neshiem, L. S. Hibbard and K. G. Mann, *Proc. Natl. Acad. Sci. U.S.A*, 78 (1981) 162.
74 A. H. Goodall, G. Kemble, D. P. O'Brien and E. Rawlings, *Blood*, 59 (1982) 664.
75 T. C. Wun, L. Ossowski and E. Reich, *J. Biol. Chem.*, 257 (1982) 7262.
76 M. Belew, T. T. Yif, L. A. Andersson and R. Ehrnstrom, *Anal. Biochem.*, 164 (1987) 457.
77 S. Krishnan, M. A. Vijayalakshmi and I. Greahel, *J. Chromatogr.*, 397 (1987) 339.
78 S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 370 (1986) 315.
79 E. Pungor, N. B. Afeyan, N. F. Gordon and C. L. Cooney, *Biotechnology*, 5 (1987) 604.
80 C. Vincent, *Biofutur Technoscope*, No. 20, May (1988) 1.
81 D. Adamski-Medda, Q. T. Nguyen and E. Dellacherie, *J. Membr. Sci.*, 9 (1981) 337.
82 T. P. Choe, P. Masse and A. Verdier, *Biotechnol. Lett.*, 3 (1986) 163.
83 J. H. T. Luong, A. L. Nguyen and K. L. Male, *Biotechnology*, 5 (1987) 564.
84 I. N. Topchieva, *Russ. Chem. Rev. (Engl. Transl.)*, 49 (1980) 260.
85 A. F. Bückmann, M. Morr and G. Johansson, *Makromol. Chem.*, 182 (1981) 1379.
86 E. Hackenthal, R. Hackenthal and U. Hilgengeldt, *Biochim. Biophys. Acta*, 522 (1978) 561.
87 K. Yamamoto, N. Katsuda and K. Kato, *Eur. J. Biochem.*, 92 (1978) 499.
88 S. M. M. Basha, M. N. Horst, F. W. Bazer and P. M. Roberts, *Arch. Biochem. Biophys.*, 185 (1978) 174.
89 C. Kettner, J. Rodriguez-Absi, G. I. Glover and J. M. Prescott, *Arch. Biochem. Biophys.*, 162 (1974) 56.
90 K. H. Rohm, *Hoppe Seyler's Z. Physiol. Chem.*, 363 (1982) 641.
91 T. Tosa, T. Sato, R. Sano, K. Yamamoto, Y. Matuo and I. Chibata, *Biochim. Biophys. Acta*, 334 (1974) 1.
92 G. Oshima and K. Nagasawa, *J. Biochem.*, 81 (1977) 1285.
93 L. B. Cueni, *Anal. Biochem.*, 107 (1980) 341.
94 Y. Narahashi, K. Yoda and S. Honda, *J. Biochem.*, 82 (1977) 615.
95 T. Plummer and M. Y. Hurnitz, *J. Biol. Chem.*, 253 (1978) 3907.
96 S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 329 (1985) 165.
97 R. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
98 J. E. Cawston and G. Murphy, *Methods Enzymol.*, 80 (1981) 711.
99 C. Gillet, Y. Ecckhont and G. Vaes, *FEBS Lett.*, 74 (1977) 126.
100 R. M. Metrione, *Biochim. Biophys. Acta*, 526 (1978) 531.
101 Y. Legrand, G. Pignaud, J. P. Caen, B. Robert and L. Robert, *Biochem. Biophys. Res. Commun.*, 63 (1975) 224.
102 K. Katagiri, T. Takeuchi, K. Taniguchi and M. Sasaki, *Anal. Biochem.*, 86 (1978) 159.

103 F. A. Pitlick and Y. Nemerson, *Methods Enzymol.*, 45 (1976) 37.
104 R. Radcliffe and Y. Nemerson, *Methods Enzymol.*, 45 (1976) 49.
105 J. H. Griffin and C. G. Cochrane, *Methods Enzymol.*, 45 (1976) 56.
106 K. Takahiko, K. Hisao and E. W. Davie, *Methods Enzymol.*, 45 (1976) 65.
107 K. Fujikawa and E. W. Davie, *Methods Enzymol.*, 45 (1976) 89.
108 H. Suda, K. Yamamoto, T. Aoyagi and M. Umezawa, *Biochim. Biophys. Acta*, 616 (1980) 60.
109 R. W. Colman and A. Bagdasarian, *Methods Enzymol.*, 45 (1976) 303.
110 B. Geiger and H. Fritz, *Methods Enzymol.*, 80 (1981) 466.
111 F. Fiedler, E. Fink, H. Tschesche and H. Futz, *Methods Enzymol.*, 80 (1981) 493.
112 E. Hackenthal, *Biochim. Biophys. Acta*, 522 (1978) 563.
113 B. Holmquist, *Biochemistry*, 16 (1977) 4591.
114 P. F. Fox, J. R. Whitaker and P. A. O'Leary, *Biochem. J.*, 161 (1977) 389.
115 J. Turková, K. Bláha, J. Horáček, J. Vajtner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
116 G. E. Sieffring and K. C. Robbins, *Methods Enzymol.*, 45 (1976) 257.
117 T. Yoshimoto, M. Fischl, R. C. Orlowski and R. Walter, *J. Biol. Chem.*, 253 (1978) 3708.
118 G. Feinstein and A. Janoff, *Biochim. Biophys. Acta*, 403 (1975) 477.
119 E. J. B. Fodor, H. Ako and K. A. Walsh, *Biochemistry*, 14 (1975) 4923.
120 D. Y. Twumasi and I. E. Liener, *J. Biol. Chem.*, 252 (1977) 1917.
121 U. Kašhe, K. Buchholz and B. Galunsky, *J. Chromatogr.*, 216 (1981) 169.
122 H. Yokosawa, T. Inagami and E. Haas, *Biochem. Biophys. Res. Commun.*, 83 (1978) 306.
123 T. Matoba, K. Murakami and T. Inagami, *Biochim. Biophys. Acta*, 526 (1978) 560.
124 H. J. Chou, J. H. Sharper and R. I. Gregerman, *Biochim. Biophys. Acta*, 524 (1978) 123.
125 T. Morita, H. Igarashi and S. Iwanaga, *Methods Enzymol.*, 80 (1981) 311.
126 K. W. Jackson, N. Esmon and J. Tang, *Methods Enzymol.*, 80 (1981) 387.
127 V. K. Akparov and V. M. Stepanov, *J. Chromatogr.*, 155 (1978) 329.
128 W. Holleman and L. J. Weiss, *J. Biol. Chem.*, 251 (1978) 1691.
129 K. Nishioka, *Eur. J. Biochem.*, 85 (1978) 137.
130 S. Ghodbane and G. Guiochon, *J. Chromatogr.*, 444 (1988) 275.
131 T. A. Anderson, *J. Chromatogr.*, 326 (1985) 33.
132 X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55.
133 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 133 (1983) 251.
134 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Biochem.*, 52 (1980) 177.
135 K. Unger, *Methods Enzymol.*, 104 (1984) 154.
136 K. H. Rohm, *Hoppe-Seyler's Z. Physiol. Chem.*, 363 (1982) 641.
137 F. E. Regnier, *Methods Enzymol.*, 104 (1984) 170.
138 M. L. Heinitz, L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 173.
139 J. S. Richey, *Methods Enzymol.*, 104 (1984) 223.
140 M. T. W. Hearn, A. N. Hodder and M. I. Aguilar, *J. Chromatogr.*, 443 (1988) 97.
141 E. Kopetzki and K. D. Entian, *Anal. Biochem.*, 121 (1982) 181.
142 J. H. L. Franssen, T. Janssen-Claessen and G. Van Diesjen, *Biochim. Biophys. Acta*, 747 (1983) 186.
143 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190.
144 R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 81.
145 D. P. Lee, *J. Chromatogr.*, 443 (1988) 143.
146 M. J. O'Hare, M. W. Capp, E. C. Nice, N. H. C. Cooke and B. G. Archer, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, p. 161.
147 K. Titani, J. Sasagawa, K. Resing and K. A. Walsh, *Anal. Biochem.*, 123 (1982) 6552.
148 M. W. Berchtold, K. J. Wilson and C. W. Heiznann, *Biochemistry*, 21 (1982) 6552.
149 M. Rubinstein, *Anal. Biochem.*, 98 (1979) 1.
150 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
151 M. T. W. Hearn and B. Greco, *J. Chromatogr.*, 266 (1983) 75.
152 W. G. Burton, K. D. Nugent, T. K. Slattery, B. R. Summers and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 363.
153 K. D. Nugent, W. G. Burton, T. K. Slattery, B. F. Johnson and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
154 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.

155 R. J. Linhardt, *Appl. Biochem. Biotechnol.*, 11 (1985) 49.
156 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
157 J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, *J. Chromatogr.*, 317 (1984) 141.
158 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1984) 519.
159 J. P. Chang, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
160 L. A. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 73.
161 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
162 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
163 S. Hjertén, K. Yao, Z.-Q. Liu, D. Yang and B.-L. Wu, *J. Chromatogr.*, 354 (1986) 203.
164 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 360 (1986) 260.
165 M. L. Heinitz, L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 173.
166 Y. Kato, *Adv. Chromatogr.*, 26 (1987) 97.
167 G. Fassina and I. M. Chaiken, *Adv. Chromatogr.*, 27 (1987) 247.
168 P. O. Larsson, *Methods Enzymol.*, 104 (1984) 212.
169 P. O. Olsson, *Biol. Chem. Hoppe-Seyler*, 368 (1987) 767.
170 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 216 (1981) 175.
171 S. P. Colowick and O. Kaplan, *Methods Enzymol.*, 80, Sect. I (1981) 3.
172 J. Turková, K. Bláha, J. Horáček, J. Vajčner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
173 S. Sasaki, S. Kobayashi, S. Kiryu and M. Sugiura, *Chem. Pharm. Bull.*, 33 (1985) 746.
174 S. Kobayashi, H. Endo, M. Sasaki, S. Kiryu and M. Sugiura, *Chem. Pharm. Bull.*, 33 (1985) 697.
175 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Biochem.*, 52 (1980) 177.
176 G. H. Caughey, N. F. Viro, J. Ramachandran, S. C. Lazarus, D. B. Borson and J. A. Nadel, *Arch. Biochem. Biophys.*, 258 (1987) 555.
177 K. Mizuno and H. Matsuo, *Nature (London)*, 309 (1984) 558.
178 B. Dahlmann, L. Kuehn and H. Reinauer, *FEBS. Lett.*, 160 (1983) 243.
179 W. E. Keene, M. G. Petitt, S. Allen and J. H. Mekerrow, *J. Exp. Med.*, 163 (1986) 536.
180 J. L. Seltzer, M. L. Eschbach and A. Z. Eisen, *J. Chromatogr.*, 326 (1985) 147.
181 P. B. Wolfe, P. Silver and W. Wickner, *J. Biol. Chem.*, 257 (1982) 7898.
182 K. Tanaka, N. Ikegaki and A. Ichihara, *J. Biol. Chem.*, 259 (1984) 5937.
183 D. J. Buttle and A. J. Barret, *Biochem. J.*, 223 (1984) 81.
184 C. Barthomeuf, H. Pourrat and A. Pourrat, *Biotechnol. Tech.*, 2 (1988) 29.
185 G. E. Maret and J. L. Fauchere, *Anal. Biochem.*, 172 (1988) 248.
186a W. Machleidt, J. Otto and E. Wachter, *Methods Enzymol.*, 47 (1977) 210.
186b A. Kanamori, N. Seno and I. Matsumoto, *Chem. Pharm. Bull.*, 35 (1987) 3783.
187 K. Titani, J. Sasagawa, K. Resing and K. A. Walsh, *Anal. Biochem.*, 123 (1982) 408.
188 P. J. Padkield and R. M. Case, *Anal. Biochem.*, 104 (1984) 416.
189 C. T. Carilli, L. C. Wallace, L. H. Smith, H. A. Wong and J. A. Lewicki, *J. Chromatogr.*, 444 (1988) 203.
190 M. Heeb and O. Gabriel, *Methods Enzymol.*, 104 (1984) 416.
191 B. J. Radola, in J. G. Radola (Editor), *Isoelectric Focusing*, Butterworth, London, 1975, p. 145.
192 B. J. Radola, *Methods Enzymol.*, 104 (1984) 256.
193 *Isoelectric Focusing, Principles and Methods*, Pharmacia Fine Chemicals, Uppsala, 1981.
194 F. Wagner, *Methods Enzymol.*, 158 (1988) 21.
195 E. A. Lance, C. W. Rhodes, III, and R. Nakon, *Anal. Biochem.*, 33 (1983) 492.
196 J. M. Prescott, F. W. Wagner, B. Holmquist and B. L. Vallee, *Biochim. Biophys. Res. Commun.*, 114 (1983) 646.
197 P. Buenning and J. F. Riordan, *Bioinorg. Chem.*, 24 (1985) 183.
198 J. D. McConn, *J. Biol. Chem.*, 239 (1964) 3706.
199 J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, 235 (1960) 390.
200 D. W. Auld and B. Holmquist, *Biochemistry*, 13 (1974) 4355.
201 E. Winsterberger, H. Neurath, T. L. Coombs and B. L. Vallee, *Biochemistry*, 4 (1965) 1526.
202 H. Hanson and M. Frohne, *Methods Enzymol.*, 44 (1980) 504.
203 W. D. Behnke and B. L. Vallee, *Proc. Natl. Acad. Sci. USA*, 69 (1972) 2442.
204 K. Breddam, T. J. Bazzone, B. Holmquist and B. L. Vallee, *Biochemistry*, 18 (1979) 1563.
205 B. Holmquist and B. L. Vallee, *J. Biol. Chem.*, 249 (1974) 4601.
206 J. W. Kusiak, J. M. Quirk and R. O. Brady, *J. Biol. Chem.*, 253 (1978) 184.
207 R. Marusyk and A. Sergeant, *Anal. Biochem.*, 105 (1980) 403.

208 H. Görish, *Anal. Biochem.*, 173 (1988) 393.
209 G. L. Gilliand and D. R. Davies, *Methods Enzymol.*, 104 (1984) 370.
210 K. M. Gernet, R. Smith and D. C. Carter, *Anal. Biochem.*, 168 (1988) 141.
211 L. Orstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
212 B. J. Davies, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
213 C. Vincent, *Biofutur Technoscope*, No. 9, February (1987) 1.
214 P. J. Blackshear, *Methods Enzymol.*, 104 (1984) 237.
215 P. H. O'Farrel, *J. Biol. Chem.*, 250 (1975) 4007.
216 N. G. Anderson and L. Anderson, *Clin. Chem.*, 28 (1982) 739.
217 R. P. Tracy and D. S. Young, in J. E. Celis and R. Bravo (Editors), *Two Dimensional Gel Electrophoresis*, Academic Press, New York, 1984, p. 193.
218 P. Blangarin and J. J. Madjar, in M. M. Galteau and G. Siest (Editors), *Recent Progress in 2-Dimensional Gel Electrophoresis*, University of Nancy, Nancy, 1986, p. 25.
219 D. F. Hochstrasser, V. Angsburger, M. Funk, R. Appel, C. Pellegrini and A. F. Muller, *Electrophoresis*, 7 (1986) 505.
220 J. J. Madjar, M. Arpin and J. P. Reboud, *Anal. Biochem.*, 83 (1977) 304.
221 P. G. Righetti, G. Tudor and E. Gianazza, *J. Biochem. Biophys. Methods*, 6 (1982) 219.
223 D. F. Hochstrasser, M. G. Harrington, A. C. Hochstrasser, M. J. Miller and C. R. Merril, *Anal. Biochem.*, 173 (1988) 424.
224 V. K. Laemmli, *Nature (London)*, 277 (1970) 680.
225 D. M. Neville, *J. Biol. Chem.*, 246 (1971) 6328.
226 M. V. Kolodzeiskaia, *Lab. Delo*, 7 (1986) 426.
227 A. H. Reisner, *Methods Enzymol.*, 104 (1984) 439.
228 O. Vesterberg, L. Hansen and A. Sjosten, *Biochim. Biophys. Acta*, 491 (1977) 160.
229 C. R. Merril, D. Goldman and M. L. Keuren, *Methods Enzymol.*, 104 (1984) 441.
230 J. E. Gander, *Methods Enzymol.*, 104 (1984) 447.
231 M. J. Heeb and O. Gabriel, *Methods Enzymol.*, 104 (1984) 416.
232 H. Maeda, *Anal. Biochem.*, 92 (1979) 222.
233 H. Khalfan, R. Abuknesha and D. Robinson, *Biochem. J.*, 209 (1983) 265.
234 C. H. Evans and J. D. Ridella, *Anal. Biochem.*, 142 (1984) 411.
235 S. S. Twining, *Anal. Biochem.*, 143 (1984) 30.
236 U. Tisljar and H. W. Denker, *Anal. Biochem.*, 152 (1986) 39.
237 H. Nagase and J. F. Woessner, *Anal. Biochem.*, 107 (1980) 385.
238 A. T. Andrews, *FEBS Lett.*, 141 (1982) 207.
239 M. D. Taylor and A. T. Andrews, *FEBS Lett.*, 157 (1983) 200.
240 R. Chavira, T. J. Burnett and J. H. Hageman, *Anal. Biochem.*, 136 (1984) 446.
241 F. C. Church, D. H. Porter, G. L. Catignani and M. E. Swaisgood, *Anal. Biochem.*, 146 (1985) 343.
242 M. T. Gislow and B. C. McBride, *Anal. Biochem.*, 68 (1975) 70.
243 J. T. Dingle, A. M. J. Blow, A. J. Barrett and P. E. N. Martin, *Biochem. J.*, 167 (1977) 775.
244 M. J. Banda and Z. Werb, *Biochem. J.*, 193 (1981) 589.
245 H. S. Jensen, *Biochem. J.*, 218 (1984) 645.
246 J. C. Unkless, T. L. Ossowski, J. P. Quigley, D. B. Rifkin and E. Reich, *J. Exp. Med.*, 137 (1973) 85.
247 E. D. Sevier, *Anal. Biochem.*, 74 (1976) 592.
248 B. D. Robertson, G. E. Kwan-Lim and R. M. Maizels, *Anal. Biochem.*, 172 (1988) 284.
249 D. L. Garner, *Anal. Biochem.*, 67 (1975) 688.
250 M. Suguira, Y. Ito, K. Hirano and S. Sawaki, *Anal. Biochem.*, 81 (1977) 481.
251 M. Pacaud and J. Vriel, *Eur. J. Biochem.*, 23 (1971) 435.
252 J. S. Mort and H. Leduc, *Anal. Biochem.*, 119 (1982) 148.
253 K. R. Lynn and N. A. Clevette-Radford, *Anal. Biochem.*, 117 (1981) 280.
254 B. G. Ohlsson, B. R. Weström and B. W. Karlsson, *Anal. Biochem.*, 152 (1986) 239.
255 J. Dijhof and C. Poort, *Anal. Biochem.*, 83 (1977) 315.
256 G. Feinein and A. Janoff, *Anal. Biochem.*, 71 (1976) 358.
257 P. E. Burdett, A. E. Kipps and P. H. Whitehead, *Anal. Biochem.*, 72 (1976) 315.
258 D. Every, *Anal. Biochem.*, 116 (1981) 519.
259 P. J. Kelleher and R. L. Juliano, *Anal. Biochem.*, 136 (1984) 470.
260 M. Hofelmann, R. Kittsteiner-Eberle and P. Schreier, *Anal. Biochem.*, 128 (1983) 217.
261 P. Sinha, *Histochemistry*, 81 (1984) 217.

262 S. A. Lacks and S. S. Sprinchorn, *J. Biol. Chem.*, 255 (1980) 7467.
263 S. S. Twining, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 42 (1983) 1951.
264 H. Ansari and L. Stevens, *Soc. Gen. Microbiol. Q.*, 8 (1981) 277.
265 A. Gerther, Y. Tencer and G. Tinman, *Anal. Biochem.*, 54 (1973) 270.
266 C. W. Ward, *Anal. Biochem.*, 74 (1976) 242.
267 K. A. Killick and L. W. Wang, *Anal. Biochem.*, 106 (1980) 367.
268 C. Heussen and E. B. Doxdle, *Anal. Biochem.*, 102 (1980) 196.
269 J. Thelu, *Anal. Biochem.*, 172 (1988) 124.
270 J. G. Linner, S. A. Livesey, D. S. Harrison and A. L. Steiner, *J. Histochem.*, 34 (1986) 1123.
271 M. Wilchek and E. Bayer, *Anal. Biochem.*, 171 (1988) 1.
272 K. Yamaguchi and H. Asakawa, *Anal. Biochem.*, 172 (1988) 104.
273 A. Mazen, G. Gradwohl and G. de Murcia, *Anal. Biochem.*, 172 (1988) 39.
274 B. Durand, *Biofutur Technoscope*, No. 19, April (1983) 3.
275 D. Walker, *Biotechnol. Lab.*, December (1988) 8.
276 J. Sock and R. Rohringer, *Anal. Biochem.*, 171 (1988) 310.