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Review

Methods for chromatographic and electrophoretic separation and assay of $NADP^+$ oxidoreductases

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Abstract

The different techniques described in purification protocols for pyridine nucleotide-dependent enzymes have been reviewed, covering mainly the papers published in the past six years. Chromatography was reported in 100% of reviewed papers and among the chromatographic techniques, affinity chromatography was the most used (ca. 92%), followed by ion-exchange chromatography (ca. 79%), size-exclusion chromatography (ca. 64%) and hydrophobic chromatography (ca. 24%). Other chromatographic techniques were used infrequently. Each chromatographic technique has a different specific capacity and chemical selectivity and, therefore, the order of selection should be based on a precise knowledge of the nature of the sample and the amount of the target enzyme that it contains. Analytical electrophoresis was used in about 95% of the reviewed papers, with denaturing polyacrylamide gel electrophoresis (PAGE) being the most widely used mode (ca. 92%), followed by native PAGE (ca. 48%). The use of isoelectric focusing was reported in 14% of the papers, while preparative gel electrophoresis was used in only 8% of the cases. The use of other electrophoretic techniques was reported in only a few papers. The use of continuous enzymatic activity assay methods (spectrophotometric) was found in most papers, while high-performance liquid chromatography-based methods (discontinuous assays) were reported in only 11% of the reviewed articles.

Keywords: Reviews; $NADP^+$ oxidoreductase

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1. Introduction

Pyridine nucleotide-linked oxidoreductases form a family of enzymes, with more than 140 members [1,2], involved in catabolic or anabolic pathways acting as dehydrogenases or reductases, respectively [3,4]. Under physiological conditions, their active sites exclusively bind NAD^+ or $NADP^+$, although a reduced group (of about 30) can indistinctly accept either of these coenzymes [1].

Once extracted, each protein shows specific requirements, whose determination is critical for its adequate handling. To preserve enzymatic activity, each purification step should be optimized in relation to the type of buffer, pH, ionic strength, temperature and additives. Frequently used buffers, such as 3-(N-morpholine)propane sulfonic acid (MOPS) or hydroxyethylpiperazine ethanesulfonic acid (HEPES), interfere with the Lowry assay and alternative methods have to be used for protein determination. Piperazine-based buffers, such as HEPES, piperazine-N,N′-bis(2-ethane)sulfonic acid (PIPES), etc, form reactive radicals under certain conditions and should not be used for studying redox systems [5]. Inorganic buffers, such as borate or bicarbonate, may interfere with the assay of different enzymes; e.g., borate forms complexes with pyridine nucleotides [6]. Buffers containing metal-chelators are inadequate for metal-requiring enzymes.

To prevent microbial contamination, sodium azide is often added to the buffer at a concentration of 0.2–0.3%, which does not affect most proteins. Many enzymes lose enzymatic activity upon oxidation, which is usually restored when their thiol groups are reduced, by using 2-mercaptoethanol (5–15 m*M*) or dithiothreitol (1–5 m*M*). Addition of an inert protein, such as bovine serum albumin (BSA), is usually required to prevent losses due to unspecific adsorption to glass surfaces. Since intracellular proteases are released by cell disruption, different protease inhibitors must be added to the buffers. Fig. 1 shows a scheme of the different steps involved in a general purification protocol that is suitable for most of the $NADP^+$-linked oxidoreductases.

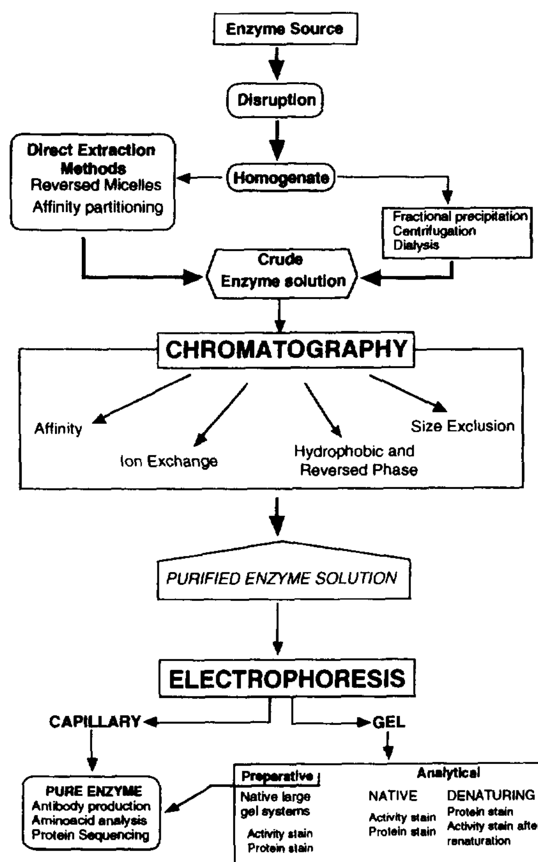

Fig. 1. General purification scheme suitable for $NADP^+$-dependent enzymes.

2. Extraction

The complexity of most biological samples accounts for the need for some pre-treatments to achieve good separations. Cell disruption is usually the first step for obtaining intracellular enzymes. Several methods are available, including homogenization, sonication, French press, grinding, vortexing with glass beads and lysis with detergents, organic solvents or osmotic shocks [7–12], with instruments available from different suppliers. Fractional precipitation by ammonium sulfate or heat treatments, followed by centrifugation or filtration are used to obtain a clear enzyme preparation. Dialysis or gel permeation are carried out for buffer exchange. Actually, several of these pre-treatments are required before chromatography or electrophoresis is carried out. Reversed micelles and affinity partitioning have recently been developed for direct extraction of intracellular enzymes from homogenates.

Reversed micelles are aggregates of surfactant molecules with a core of water molecules dispersed into an organic phase. Proteins are incorporated into the micelles by liquid–liquid extraction, but conditions should be optimized to get maximal phase transfer. Since electrostatic interactions govern the distribution of proteins between micellar and conjugate aqueous phases, pH and ionic strength of the aqueous phase must be optimized. The purification of several proteins by reversed micelles has been reviewed by Dekker et al. [13]. Isocitrate-, β-hydroxybutyrate and glucose-6-phosphate dehydrogenases were purified from *Azotobacter vinelandii* cells using reversed cetyltrimethylammonium bromide (CTAB) micelles [14]. Over 100% recovery and six-fold purification were obtained for isocitrate and β-hydroxybutyrate dehydrogenases, while the large size of glucose-6-phosphate dehydrogenase (G6PDH) ($\sim 200 \cdot 10^3$ rel. mol. mass) rendered it inadequate for this procedure.

Two-phase partitioning in aqueous systems containing hydrophilic polymers (such as polyethyleneglycol (PEG) or dextran) and salts, fractionate protein mixtures as an alternative to extraction from homogenates [15]. Selectivity increases if an affinity ligand is covalently bound to one of the phase-former polymers, thus allowing the specific extraction of the target enzyme. Triazine dyes have been used for large-scale extraction of many proteins and enzymes, including several $NADP^+$-linked dehydrogenases [16–19]. Affinity partitioning with Procion Red HE3b bound to PEG was used for large-scale extraction of formate dehydrogenase from *Candida boidinii* homogenates [20,21]. Direct extraction of G6PDH from rat erythrocyte hemolysates was reported by Delgado et al. [22] using Cibacron Blue F3GA–PEG–dextran two-phase systems, with 40-fold purification and 60% recovery.

New affinity separations using perfluorocarbon emulsions (PERCAS) have been described recently by McCreath et al. [23]. Using Procion Red H-E7b as the affinity dye ligand, they reported direct extraction of G6PDH from baker's yeast homogenates, achieving eighteen-fold purification and sample clarification in a single step. Similar results were obtained using expanded bed affinity chromatography (EBAC), although PERCAS produced about a 2.25-fold higher yield than EBAC [23]. The affinity techniques previously described are suitable for "dirty samples" in which particulate matter is present, such as cell homogenates. Inclusion of these highly selective purification steps in the early stages of the purification protocol could significantly improve the process.

3. Chromatographic procedures

Chromatography is a widely used method for protein purification. Previous chromatographic schemes contained long and complicated steps, required large homogenate volumes and resulted in low final yields. A plethora of books, reviews and articles dealing with the applications of chromatographic methods in protein chemistry have been published but are outside the scope of this review.

The soft supports for conventional liquid chromatography (cellulose, agarose or dextran) have severe drawbacks, such as bacterial degradability, low mechanical stability, large particle size, poor resolution and large separation times and they must be used in a cold room to diminish enzyme inactivation. In spite of such disadvantages, recently published purifications of $NADP^+$-dependent enzymes use mainly these soft gels [24–27]. Obviously, such supports are not suitable for HPLC, which is essen-

tially free of the above-mentioned problems and highly valuable for unstable proteins. The use of HPLC for protein chemistry, starting in the late 1970s, was severely limited by the slow development of proper stationary phases, and a considerable amount of effort was made to improve them. The new supports avoid the irreversible adsorption of proteins and withstand the high pressures and flow-rates usual in HPLC. Nowadays, modern HPLC systems separate complex protein mixtures much faster than the outdated LC systems, with high recovery being obtained while operating at room temperature.

Silica [28,29] and polymer-based supports [30,31] are regularly used for protein separation by HPLC. These packings can be prepared in particle sizes from 5 to 50 μm and with pore diameters from 70 to beyond 1000 Å [32], and are stable in the pH range 2–7.5 for silica and 2–12 for polymeric supports. The supports can be derivatized and prepared for most chromatographic modes used with $NADP^+$-dependent enzymes, such as size-exlusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and affinity chromatography (AC) [33,34]. Separations of complex protein mixtures by porous packings are one order of magnitude faster (<1 h) than by soft gels, with very high enzyme recovery in most cases. Due to the high cost of such columns, the use of guard-columns is highly recommended.

Although non-porous packings were explored in the early stages of HPLC develpment, their use was not generalized. In 1986, Unger and co-workers [35,36] showed that small non-porous packings are very useful for high-speed separations of proteins. Since they lack pores, no diffusion is possible within the particles and separations are carried out one order of magnitude faster (few min) than with porous HPLC supports [37]. There are several types of non-porous supports based on synthetic hydrophilic polymers, cross-linked polystyrenes or silica, available for separation of proteins by IEC, HIC or by AC. The POROS® support from PerSeptive Biosystems (Cambridge, MA, USA) and the TSK-NPR® family from Tosoh (Tokyo, Japan) are useful for the separation of biopolymers by the new technique of 'perfusion chromatography', a methodology reviewed in-depth by Hashimoto [38] and by Afeyan and co-workers [39,40]. No publication has been found in the recent literature that used perfusion chromatography for the purification of $NADP^+$-linked enzymes.

Corrosion in wetted parts of the equipment and low protein recoveries were frequently reported in early papers describing the use of HPLC for protein purification. The buffers used for proteins contain halides which corrode the stainless steel components of the equipment, leaching out traces of metal ions which could alter different proteins. The low recoveries observed could be attributed to irreversible adsorption of proteins to the surface of tubing and other wetted stainless steel components. During the last decade, manufacturers have paid particular attention to the design of HPLC equipment with sample loops, pump heads, tubing, columns, frits, fraction collectors and other wettable parts made with inert materials (Teflon, Kel-F, polyethylene, PEEK, etc.) instead of stainless steel and, in general, suitable for use in a cold room. They are named "biocompatible" and widely overcome the problems stated above; biocompatible systems are available from several HPLC makers: FPLC from Pharmacia, Iso-Pure from Perkin-Elmer, Waters 650 from Waters and the Nouveau series from Beckman Instruments. Thus, the chromatographer now has available a wide range of automated HPLC equipment which is capable of continuously monitoring the eluent from the chromatographic column and of carrying out protein purification from complex mixtures, within a time scale of h, instead of days, as is usual for conventional LC.

Nevertheless, the term "biocompatibility" is somewhat controversial. Obviously, such equipment is useful and can be highly recommended to achieve fast protein purification (one day's work) with high recovery and excellent purification rates. Upon the introduction of "biocompatible" instruments into the market, biochemists are faced with the decision of whether to get such an apparatus or to continue with the classical stainless steel HPLC instruments. If no corrosion problems exist and no significant adsorption or inactivation of proteins is detected, the use of classic HPLC equipment should not be excluded. We have reported a method for the purification and quantitation of glutamine synthetase by high-performance immunoaffinity chromatography using a

HPLC system and a stainless steel column [41]. The single step method with cell-free extracts yielded electrophoretically homogeneous enzyme in less than 25 min, with a 70% recovery of the enzyme activity loaded into the column and a 100% recovery of protein. Quantitative results obtained were similar to those obtained by rocket immunoelectrophoresis [41].

3.1. Affinity chromatographic techniques

In the AC methodology, particular molecules are isolated based on their biospecific interaction with a complementary immobilized molecule, the affinity ligand. Among the chromatographic techniques, AC offers the greatest specificity and selectivity for enzyme isolation, usually being carried out at the final purification stages, mainly due to the cost and lability of the affinity media. Thus, the use of very clear samples is highly recommended for AC. The principles and applications of AC have been reviewed in detail [16,17,44].

Many affinity media are available, that have soft, semi-rigid or rigid supports and are in packed columns or in bulk form. Several reactions have been developed to immobilize affinity ligands to the supports [16,44,45]. The supports for AC contain activated groups, such as epoxy (Waters, Beckman), hydroxyl (Toyo Soda), tresyl (Pierce) and nitrophenyl or N-hydroxysuccinimide (BioRad), which form stable covalent linkages with a variety of affinity ligands through appropriate functional groups, such as thiol, hydroxyl and primary or secondary amine. Access of the coupled ligand to the enzyme binding site is improved by intercalating spacer arms (1,6-diaminohexane or 1,4-diaminobutane) between the ligand and the support backbone [16,44].

To minimize the denaturation of enzyme molecules, conditions for all steps of the AC process, including disruption of enzyme–ligand interactions, must be mild enough. Elution can be accomplished either by non-specific or specific means, using gradient, pulse or isocratic modes. Changes in temperature, ionic strength, pH and concentrations of chaotropic ions or organic solvents in the mobile phase are suitable for non-specific desorption of enzymes. For specific elution, an appropriate counter-ligand is added to the mobile phase.

Two families of affinity ligands have been used mainly with $NADP^+$-linked enzymes: (i) specific group ligands, including adenine nucleotide derivatives [44,45] or triazine dyes [18,47,48], and (ii) specific ligands, such as antibodies [46]. Their ability to retain a certain enzyme depends on whether the geometrical orientations of the ligand fit the enzyme binding site properly [49].

Adenine nucleotide derivatives, bound to supports through either the N_6 [50] or the C_8 [51] adenine ring position, the hydroxyl groups of the ribose ring [52] or the phosphate group [53], have been used for conventional AC of many pyridine nucleotide-dependent enzymes [16,44]. Among them, 8-(6-aminohexyl)amino-2′-phosphoadenosine diphosphoribose (C8ATPR) and 2′,5′-ADP have been successfully applied to isolate several $NADP^+$-linked enzymes [55,56]. High-performance liquid affinity chromatography (HPLAC) combines the biospecificity of conventional AC with the speed of operation, resolution and sensitive detection of HPLC techniques [29]. The HPLAC methodology allows multiple runs in a day, with very good reproducibility, high recovery and the process can be performed at room temperature in most cases. We have used a stainless steel pre-packed epoxy-activated silica Ultraffinity® column (5 × 0.45 cm I.D.) from Beckman, for one-step purification of glutathione reductase (GSSGRase) and G6PDH from different cell-free extracts [57]. The column was easily derivatized by recirculating a solution of the ligand C8ATPR, previously coupled to 1,6-diaminohexane as the spacer arm. Fig. 2 shows the elution pattern for GSSGRase and G6PDH from a crude extract of baker's yeast and the results summarized in Table 1 indicate the excellent behaviour of such an affinity support towards these enzymes.

Triazine and other structurally related dyes have been also used for the purification of several enzymes by HPLAC [58–61], due to their affinity for the dinucleotide fold of $NADP^+$-dependent enzymes [62]. In 1978, Watson et al. [48] reported that immobilized Procion Red HE3b retained $NADP^+$-dependent dehydrogenases more efficiently than NAD^+-linked enzymes, which interacted preferentially with Cibacron Blue F3GA. Dyes are less

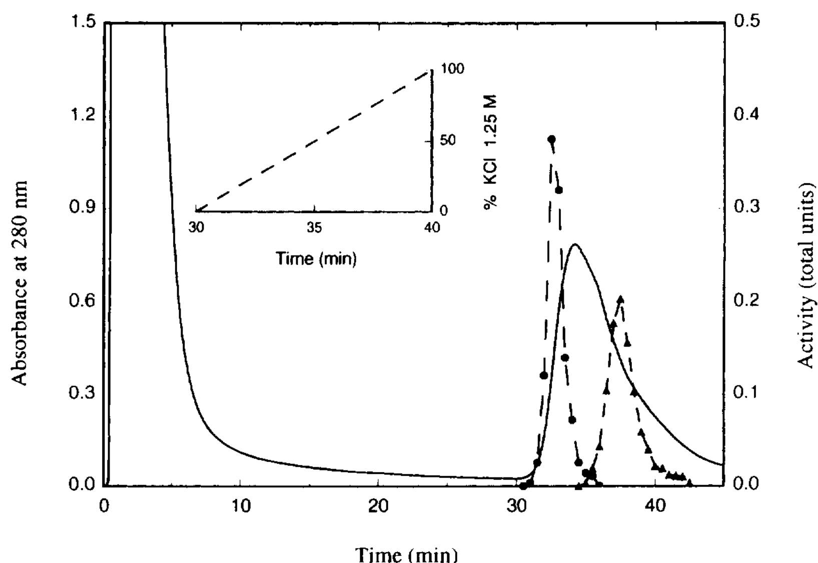

Fig. 2. HPLAC of a baker's yeast cell-free extract. A sample (2 ml) was injected onto an Ultraffinity-EP (5×0.45 cm I.D.) column derivatized with C8ATPR. Starting buffer was 250 m*M* MOPS, pH 7.5, containing 1 m*M* EDTA. Elution was carried out with a linear potassium chloride gradient (0–1.25 *M*) in starting buffer. The solid line represents the absorbance at 280 nm; (●) GSSGRase activity; (▲) G6PDH activity. Flow-rate was 1 ml/min and pressure was 400 p.s.i. ($2.76 \cdot 10^6$ Pa). From Ref. [57] with permission.

expensive than nucleotide analogues, show higher binding capacity (~ten-fold), are widely available and can be easily immobilized. Although the broader specificity of dyes could be considered disadvantageous [60], it allows such specific group ligands to interact with a wide number of $NADP^+$-dependent enzymes. The specificity of affinity elution is highly improved by washing the column with a pulse of NAD^+ to elute enzymes with dual cofactor requirements before eluting with $NADP^+$ [63]. Most affinity supports specific for $NADP^+$-dependent enzymes display loading capacities of up to 10 mg protein/g.

AC can be the only step for purification of $NADP^+$-linked oxidoreductases [64]. López-Barea

Table 1
Purification of GSSGRase and G6PDH from different cell-free extracts

Cell free extracts (loading buffer)	GSSGRase		G6PDH	
	Recovery (%)	Purification (fold)	Recovery (%)	Purification (fold)
Baker's yeast				
MOPS 250 m*M*, pH 7.5	90	104	118	35
Fish liver				
MOPS 250 m*M*, pH 7.5	unbound	unbound	59	57
MOPS 50 m*M*, pH 7.5	62	28	63	22
MOPS 25 m*M*, pH 7.0	70	19	69	19
Rabbit hemolysate				
MOPS 250 m*M*, pH 7.5	unbound	unbound	66	502
MOPS 25 m*M*, pH 7.0	77	142	67	440

Note: A 2.0-ml sample of each cell-free extract was loaded into the Ultraffinity-EP (5×0.45 cm I.D.) column derivatized with C8ATPR. The recovery is expressed as percentage of the initial activity loaded. The purification is expressed as the ratio of the specific activity of the peak fraction/specific activity of the cell-free extract. From Ref. [57] with permission.

and Lee used two sequential AC steps with pyridine nucleotide analogues (2′,5′-ADP and C8ATPR) for the purification of GSSGRase, with 66% yield and 4695-fold purification for the mouse liver enzyme [55] and 63% recovery and 2632-fold purification for the *E. coli* enzyme [65]. *Pseudomonas putida* morphine dehydrogenase was purified by Bruce et al. [66] using dye AC in Mimetic Orange 3 and Mimetic Red 2 (Affinity Chromatography Ltd, Isle of Man, UK) as the only chromatographic steps with an 84% yield and a 1216-fold purification; the enzyme was eluted with a salt gradient, since specific elution with NADH or NADPH was unsuccessful [66]. Tandem dye–ligand AC was used by Hey and Dean [64] for the isolation of G6PDH from *Leuconostoc mesenteroides*; the enzyme was purified almost 56-fold in a two-step tandem chromatographic system using Matrex Red Purple and Matrex Red Orange B (Amicon) and eluting with a 50 μM $NADP^+$ pulse [64].

Phospho-cellulose is useful for enzymes with affinity towards phosphorylated substrates [67,68]. Takagahara et al. [68] used phospho-cellulose for the purification of yeast G6PDH, 6-phosphogluconate dehydrogenase and GSSGRase. Orthophosphate is a competitive inhibitor with respect to either $NADP^+$ or 6-phosphogluconate in these enzymes, which were sequentially eluted by successive additions of their respective substrates [68].

Higher selectivity and specificity is achieved by using antibodies as immobilized ligands, which adds the advantages of immunochemical reactions to the chromatographic process. Polyclonal or monoclonal antibodies have been used to purify many proteins to electrophoretic homogeneity by a single-step immunoaffinity chromatographic procedure [41,69]. Production of monoclonal antibodies is a laborious and expensive process, and the choice depends on consideration of production cost and column half-life. Drastic elution conditions, with low-pH buffers, high concentrations of chaotropics, organics or mixtures (organics plus salts), are usually needed [41,69,70]; thus, the enzyme is usually recovered in an inactive form. Purification of G6PDH from rabbit reticulocytes by immunoaffinity chromatography was reported in 1994 by Ninfali and Marinoni [46]. Goat anti-rabbit G6PDH IgGs were immobilized, through the oxidized carbohydrate moieties, on a pre-packed 2-ml Carbolink column (Pierce). The enzyme was eluted in a fully inactive form with 0.1 *M* acetic acid in 1 *M* NaCl and was identified by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), using pure G6PDH as the standard and densitometric scan analysis, with a final recovery of 92% of the protein loaded [46].

Some selected applications of affinity chromatography are summarized in Table 2. In publications dealing with the purification of $NADP^+$-linked oxidoreductases published since 1990, at least 95% of the papers revised used AC steps. 54% of protocols utilized dyes and about 33% of them used nucleotide analogues as affinity ligands.

3.2. IEC and chromatofocusing (CF)

IEC has been applied to protein separation since early in the 1960s. Separation in IEC is achieved on the basis of either the charge or the isoelectric point of the proteins and is frequently used as an early purification step, due to the high loading capacity of supports and its ability to remove many contaminants of different electric charge. Commercial IEC supports for proteins offer excellent resolution, quantitative recovery of biological activity and short separation times. In many IEC supports, the operative pH ranges from 2 to 13 and the ion-exchange capacity is higher for polymeric macroreticular supports (1–10 mequiv./g) than for bonded-phase pellicular particles (20–100 μequiv./g). In practice, the sample loaded must be less than 5% of the ion-exchange capacity of the column, i.e. 0.2–2 mg of protein/g for pellicular supports and approximately 100 mg/g for polymeric materials. Anion-exchange supports are made with either di-ethylaminoethyl (DEAE), polyethyleneimine (PEI) or quaternary ammonium (QA) groups, and cation-exchangers contain either carboxymethyl (CM) or sulfopropyl (SP) groups. Based on their pH-dependent ionization, DEAE, PEI and CM exchangers are considered "weak" and QA and SP "strong". Protein elution from IEC columns is usually carried out by salt gradients (up to 2 *M*). In some cases, the polymeric support matrix exhibits a certain hydrophobicity, and non-specific retention of proteins may occur. Such unwanted interactions can be avoided by using a convenient concentration of chaotropic salts in the loading buffer [71].

Chromatofocusing was developed by Sluyterman

Table 2
Affinity ligands useful for the chromatography of $NADP^+$-dependent oxidoreductases

Affinity ligand	Enzyme/source	Elution type	Purification factor[a]	Recovery (%)[a]	Reference
Cibacron Blue F3GA	17-β-Hydroxysteroid dehydrogenase/Human placenta	35 μ*M* $NADP^+$ pulse	7.8	78	[42]
	Homoserine dehydrogenase/*Corynebacterium glutamicum*	2 m*M* $NADP^+$ pulse	95	61.9	[27]
	Glutamate dehydrogenase/*Paracoccus denitrificans*	1 *M* KCl pulse	9.25	77.3	[43]
	Carbonyl reductase/*Candida macedoniensis*	0–1 *M* NaCl gradient	2.9	77	[54]
	D-Xylose dehydrogenase/Pig liver	1 m*M* $NADP^+$ pulse	4.6	68	[101]
	Lysine-ketoglutarate reductase/*Zea mays*	0–15 m*M* $NADP^+$ gradient	2.8	33	[158]
	Cinnamyl alcohol dehydrogenase/*Nicotiana tabacum*	0–4 m*M* $NADP^+$ gradient	77	40	[63]
	2′-Hydroxydaidzein oxidoreductase/Soybean cells	3.5 m*M* $NADP^+$ pulse	17.7	26	[166]
Procion Red HE3b	Glycerol dehydrogenase/*Aspergillus niger*	0–3 m*M* $NADP^+$ gradient	28	75	[25]
	Carbonyl reductase/Rabbit kidney	0–1.2 *M* KCl gradient	3.6	50	[194]
	DMIRase/Pea seedlings	1 m*M* NADPH pulse	2.7	36	[122]
	Alcohol dehydrogenase/*Thermoanaerobacter ethanolicus*	0.5 m*M* $NADP^+$ pulse	24	73	[195]
	Morphine dehydrogenase/*Pseudomonas putida*	0.1 *M* NaCl pulse	11.3	84	[66]
	Glutamate dehydrogenase/*Neurospora crassa*	1 m*M* NADPH pulse	4.6	40	[48]
	Glutathione reductase/Horse liver	0–0.6 *M* NH_4Cl gradient	8.3	90	[196]
	Aldehyde reductase/Rat liver	5 m*M* NADPH pulse	95	58	[197]
Orange A	Morphine dehydrogenase/*Pseudomonas putida*	0.8 *M* KCl pulse	108	138	[66]
	Pyrroline-5-carboxylate reductase/Soybean nodules	0.1 m*M* NADPH pulse	124	61	[198]
	Aldose reductase/Bovine kidney	0.2 m*M* NADPH pulse	Separation of unactive and active forms		[199]
	G6PDH/*Leuconostoc mesenteroides*	50 μ*M* $NADP^+$ pulse	55.6	73	[64]

Reactive Brown	G6PDH/*Acetobacter hansenii*	0.15–1.0 *M* KCl gradient	3.8	74	[3]
2;5-ADP	GSSGRase/Easter white pine needle	10 m*M* $NADP^+$ pulse	38.1	103	[162]
	GSSGRase/*Medicago sativa*	10 m*M* $NADP^+$ pulse	101	25	[200]
	GSSGRase/*Onobrychis vicifolia*		76	64	[200]
	GSSGRase/*Rhodospirillum rubrum*	0–50 μM NADPH gradient	107	n.r.[b]	[201]
	GSSGRase/Pea seedlings	0–10 m*M* $NADP^+$ gradient	43	95	[161]
	GSSGRase/Horse liver	2.5 m*M* NH_4Cl pulse	134	94.5	[196]
	GSSGRase/*Escherichia coli*	0–10 m*M* $NADP^+$ gradient	958	86	[65]
	G6PDH/*Acetobacter hansenii*	20 μM $NADP^+$ pulse	1.1	64	[3]
	G6PDH/Mammalian erythrocyte	0.2 m*M* $NADP^+$ pulse	413	65	[67]
	Thioredoxin reductase/Bovine liver	1.0 *M* KCl pulse	19.3	71	[167]
	Cinnamyl alcohol dehydrogenase/*Nicotiana tabacum*	0–4.0 m*M* $NADP^+$ gradient	2.6	37.5	[63]
	6-Phosphogluconate dehydrogenase/*Zea mays*	2 m*M* $NADP^+$ pulse	46.5	83	[24]
$NADP^+$	G6PDH/Erythrocyte haemolysate	0–0.2 m*M* $NADP^+$ gradient	4571	50	[202]
	G6PDH/Yeast and human erythrocyte haemolysate	0–1.0 m*M* $NADP^+$ gradient	4000	40–75	[203]
C8ATPR	Thioredoxin reductase/Bovine liver	0–1.0 *M* KCl gradient	3.7	70	[167]
	GSSGRase/*Escherichia coli*	0–10 m*M* $NADP^+$ gradient	1.5	86	[65]
	GSSGRase/Mouse liver	0–0.5 m*M* $NADP^+$ gradient	35	86	[55]
P-Cellulose	G6PDH/Erythrocyte haemolysate	50 m*M* citrate+0.5 m*M* $NADP^+$	42	87	[67]
	G6PDH/Baker's yeast	0.2 m*M* $NADP^+$ gradient	118.7	85	[68]
	GSSGRase/Rat liver	10–280 m*M* potassium phosphate	30	68	[204]
GSSG	GSSGRase/Different sources	NaCl, 1 m*M* $NADP^+$ or 0.1 m*M* NADPH			[205–207]

[a]Reported values with respect to the sample loaded onto the column.

[b] n.r. = not reported.

and Elgersma [72,73] as a modification of IEC. In this chromatographic mode, an artificial pH gradient is created within the column and used for protein elution instead of the increased ionic strength exploited in conventional IEC. The CF technique is carried out in special supports, bearing immobilized anion-exchange groups of broad titration curves which provide inherent buffering capacity to the matrix. Mixtures of electrolytes (polybuffers), with a wide range of p*K* values, are pumped into the column and, upon interaction with the packing, generate a decreasing pH gradient which advances through the column [74,75]. This modifies the charge of proteins, releasing them from the stationary phase when the pH of the mobile phase is lower than their p*I*, but being reabsorbed if the pH of the polybuffer is higher than p*I* of the protein. Thus, the different proteins of the sample are focused within the column according to their p*I*, due to many repeated steps of absorption and desorption, and are eluted according to p*I* differences [72–75]. In addition to purification, CF is highly valuable for separation of isoenzymes and, together with IEF, is suitable for determination of their isoelectric points.

The use of different modes of IEC was reported in 79% of the purification protocols published since 1990 and consulted for the present review. DEAE was the exchanger group most frequently used (~68%), followed by QA (~23%), while other exchangers, such as CM, were reported in just 3% of the articles reviewed. CF was employed in about 8% of purification protocols. Human erythrocyte G6PDH [212] was purified 64 884-fold with 71% recovery by AC in 2′,5′-ADP-Sepharose and anion-exchange on DEAE 5PW. Fig. 3 shows the behaviour of this enzyme in the IEC–FPLC column, which allowed its separation from the bulk of hemoglobin present in such specialized cells. Separation of several horse-liver GSSGRase isoforms [196] by CF is shown in Fig. 4.

3.3. HIC and reversed-phase chromatography (RPC)

The term HIC was introduced by Hjerten to describe protein separation in weakly hydrophobic carbohydrate gel matrices [76]. HIC and RPC are similar, since in both modes solute retention derives from hydrophobic interactions with the packings, while they differ in the strength of interaction between proteins and supports. RPC uses packings with highly dense and lipophilic bonded phases, producing a very strong protein interaction. Consequently, organic solvents are required to elute the

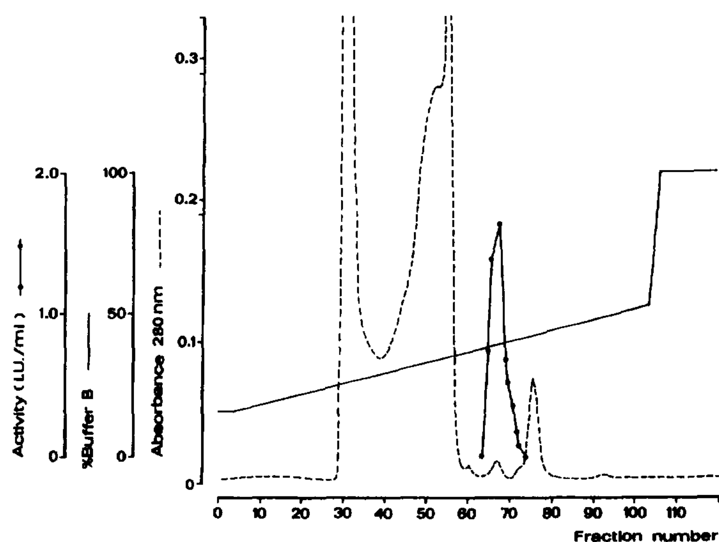

Fig. 3. Anion-exchange chromatography of human erythrocyte G6PDH on a Protein Pak-glass DEAE 5PW (7.5×0.8 cm I.D.) column. Buffer A was 5 m*M* potassium phosphate, pH 6.9, containing 1.25 m*M* EDTA and 1 m*M* aminocaproic acid. Buffer B was buffer A plus 0.5 *M* potassium chloride. Flow-rate was 0.5 ml/min and 150-μl fractions were collected; (●), G6PDH activity. From Ref. [212] with permission.

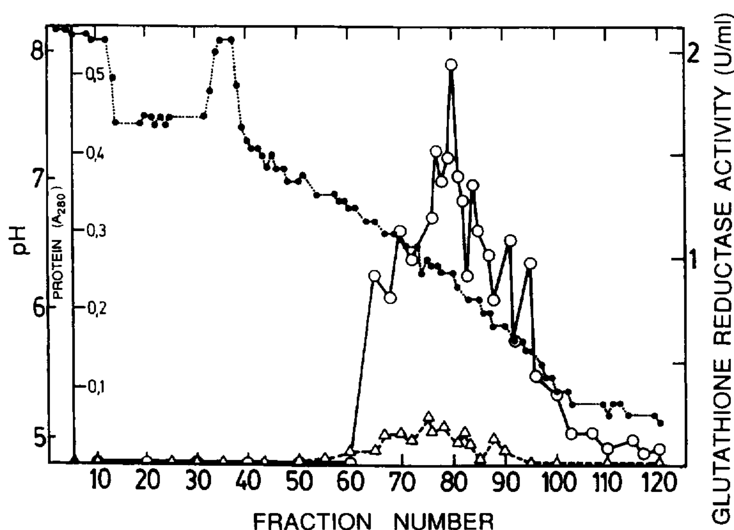

Fig. 4. Chromatofocusing of horse-liver GSSGRase. A sample of purified enzyme (0.46 mg) was loaded in a Polybuffer Exchanger 94 (30×0.5 cm I.D.) column and eluted using a pH gradient. Fractions (2 ml) were collected; (○) = enzyme activity, (●) = pH and (△) = absorbance at 280 nm. From Ref. [196] with permission.

proteins, resulting in denaturation and loss of biological activity, since renaturation is a very slow process that is not achieved in many cases [77]. The lower ligand density and hydrophobicity of the bonded phases for HIC result in mild interactions with proteins and elution is accomplished with saline buffers instead of organics. Thus, in HIC most proteins undergo little (if any) denaturation and are recovered with their intact biological activity [77].

Proteins are only retained by the HIC support at high salt concentrations (about 3 M ammonium sulfate) which favour hydrophobic binding, and elution is carried out by decreasing salt gradients in the mobile phase. Excessive hydrophobicity produces severe retention, leading to band broadening, mass loss or protein inactivation. Phenyl, butyl and polyether are useful bonded phases for HIC of proteins. Selectivity can be increased by controlling the type and concentration of salts, the pH, temperature and the gradient shape. The mild adsorption and elution conditions of HIC result in high protein recovery and provide one of the most suitable techniques for enzyme purification.

The use of HIC was reported in about 24% of papers reviewed and phenyl was the bonded phase most used for $NADP^+$-linked oxidoreductases. Fig. 5

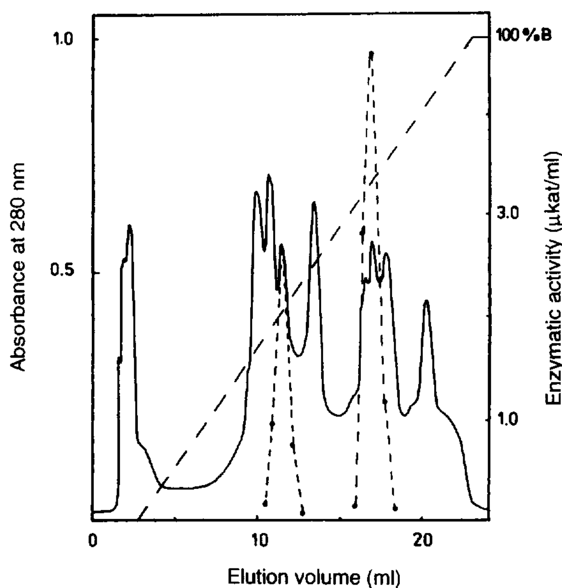

Fig. 5. Hydrophobic interaction chromatography of a *Leucosnostoc mesenteroides* crude extract after ammonium sulfate fractionation on a Phenyl Superose HR 5/5 (5×0.5 cm I.D.) column. Buffer A was 50 mM sodium phosphate, pH 7.4, with 1.7 M sodium sulfate and 1 mM EDTA, and buffer B was 50 mM sodium phosphate, pH 7.4. Flow-rate was 0.5 ml/min. (○) = G6PDH activity, (●) = LDH activity. The sample applied contained 10 mg of protein. From Ref. [78] with permission.

shows the behaviour of G6PDH from a crude extract of *Leucosnostoc mesenteroides* using a Phenyl-Superose HR 5/5 column, which produced a 63% yield and a 71-fold purification factor [78].

3.4. SEC

Although SEC has not experienced fast growth in recent years, a few new columns are introduced in each Pittsburgh Conference (the last was held in New Orleans, 5–10 March, 1995). SEC packings available for HPLC have particle sizes of 5–10 μm with pore sizes up to 10^4 Å, are stable over a wide pH range (2–13) and resist up to $4.13 \cdot 10^7$ Pa (6000 p.s.i.). SEC is often used as an initial chromatographic step to fractionate the components of a sample with minimal loss or degradation of protein. SEC is also utilized for desalting or changing the sample buffer. Sample load is low to moderate, the capacity of many packings reaching up to 1 mg protein/g support. HPLC SEC columns are often four to five-fold bigger than standard reversed-phase (25 cm×4.6 mm) columns, and ten to twenty-fold bigger than IEC columns [79]. The use of SEC as either the preparative or analytical step was reported in about 64% of protocols reviewed. Among the different SEC supports, Sephadex (ca. 34%), Sephacryl (ca. 26%) and Superose (ca. 14%) were the most frequently reported.

4. Electrophoretic methods

The differential mobility of proteins in an electric field is the basis of electrophoretic separations, widely used in biochemistry to determine purity, molecular mass and p*I* of proteins [80–82]. Modern protein electrophoresis began with the experiments of Tiselius on moving boundary electrophoresis [83,84], requiring tens of milligrams of sample for detection. Over the last decades, many efforts have been made for the development of equipment to carry out different electrophoretic modes, such as zonal [85–87], isotachophoresis [88], IEF [89–91] and CE [92–95].

4.1. Analytical gel electrophoresis

Most electrophoretic methods were initially conceived for analytical purposes [96], not being widely used at the preparative scale [97–99]. Slab gels are more popular than cylindrical gels, probably due to the possibility of running many samples in parallel. Polyacrylamide is the polymer most used for gel electrophoresis [100]. Different modes of PAGE, such as homogeneous, discontinuous or gradient systems [102,103], under either native [104,105] or denaturing conditions (SDS, SDS–urea) [106,107] or even two-dimensional systems [108], were designed to quantitate, compare and characterize enzymes. Today, gel electrophoresis is a well established routine technique in all protein laboratories. Equipment for different electrophoretic modes (at analytical or preparative scales, with horizontal or vertical formats) are commercially available from several manufacturers, such as Bio-Rad, Hoefer, CBS Scientific, Applied Biosystems, Novel Experimental Technology, Pharmacia, etc. Usually quantitation is carried out by densitometry.

Despite its potential for high resolution of complex enzyme mixtures, PAGE suffers from several pitfalls implicit to the polymerization process. Unwanted contaminants, such as polymerization initiators, unpolymerized acrylamide and acrylic acids, affect the resolution of many proteins and should be excluded. Commercial precast gels minimize many such problems, making the electrophoretic resolution of the samples more reproducible. One of the major limitations of gel electrophoresis is the Joule heating effect due to the electric field applied. The poor heat dissipation of gels only allows their use at relatively low potentials and hence is considered as a slow separation technique. In most cases, the use of anticonvective gels and cooling devices considerably improves protein resolution [109].

Although PAGE is the most used electrophoretic technique, other supports using starch [110], cellulose acetate [111], agar [112] and agarose [113] gels have been applied with many enzymes, although such alternative methods are either time-consuming, expensive or require special equipment. Agarose is less expensive than starch or other gels. Agarose gel electrophoretic separation of G6PDH isoenzymes was reported by Forbes et al. [113] for large-scale screening in clinical diagnosis using standard microscope slides; isoenzymes were resolved at room temperature in less than 45 min, using 5 μl of human hemolysate.

Affinity electrophoresis is useful for studying enzyme–ligand interactions [114]. This electrophoretic mode is analogous to AC, but requires much lower amounts of enzyme, enabling at least an approximate estimation of the enzyme–ligand dissociation constants. Several variants of affinity electrophoresis based on polyacrylamide [115], agarose or agarose–polyacrylamide gels [116] with covalently bound ligands have been developed for qualitative or quantitative purposes. Review articles are available referring to this increasingly used technique [117,118]. The simplest procedure to prepare affinity gels is to add a high molecular mass soluble polymer, with the affinity ligand covalently bound to the polymerization solution used for electrophoresis [115,119]. The interactions of several $NADP^+$-dependent dehydrogenases with Cibacron Blue F3GA using affinity electrophoresis were reported by Ticha et al. [120] in polyacrylamide gels prepared with the dye covalently bound to Dextran; electrophoretic mobility of enzymes in the affinity gels decreased with Blue Dextran concentrations.

4.2. Preparative electrophoresis

This technique has not been frequently used in the purification protocols reviewed, since separation by electric charge or p*I* is also successfully obtained in IEC, which is less complicated and faster than electrophoresis in most cases. If a pure enzyme preparation is required for antibody production or microsequencing, different modes of preparative electrophoresis, such as IEF [97] or isotachophoresis [121] are highly efficient for achieving purifications that could not be easily performed in a single-step by any separation technique. Since separation depends simultaneously on both size and charge, proteins that cannot be separated under the usual SEC, IEC or HIC methods can be resolved by this system [122,123].

A preparative electrophoretic method for detection and purification of pyridine nucleotide-linked oxidoreductases was reported in 1989 by Seymour and Lazarus [105]. Separation was carried out in 1.5 mm-thick vertical gels prepared with bisacrylylcystamine (BAC) instead of the usual cross-linker, bisacrylamide. The high N,N,N′,N′-tetraethylmethylenediamine (TEMED) concentration needed for polymerization of BAC-based gels was eliminated by pre-electrophoresis prior to sample introduction. After the bands of interest were located and excised, the crosslinks could be broken by thiols; thus, proteins containing disulfide bonds would require reoxidation to recover the native structure. A 50% recovery was achieved with this gel system for α-ketoaldonate reductase [105]. Some selected applications of gel electrophoresis are summarized in Table 3.

4.3. CE

In contrast with conventional slab gel electrophoresis, CE is suitable for on-column detection and, therefore, for real-time data analysis and automation. It is an exciting approach to fast separations, with good resolution and very minute sample requirements (few nanoliters), that began in the last decade. Several CE techniques, such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), micellar electrokinetic chromatography (MECC) and capillary gel electrophoresis (CGE), have been developed for the separation of many proteins. The potential of CE techniques to combine speed with high resolution [124,125] has sparked the interest of many scientists. CZE has similar selectivity to IEC. CIEF and CGE have applications similar to those of traditional gel electrophoresis. [126,127]. The main drawback of early CE methods was the difficulty involved in collecting enough sample for further work and its lack of sensitivity. These problems have been overcome and devices to collect fractions [128] and to increase detector sensitivity, such as isotachophoresis [129] or on-line pre-concentration with discontinuous buffers [130], have been developed [131]. The state of the art in CE technology has been reviewed in detail [126,132–136].

In some cases, CE separations of proteins exhibit poor peak resolution and low recoveries due to complex and even tenacious binding to column packings or strong adsorption to the walls of fused bare silica capillaries [131]. High-efficiency separation of proteins now can be performed using buffer additives and coated capillaries. Major developments in CE theory, equipment and applications have occurred since the first commercial products were introduced in 1988. Coated capillaries, diminishing

Table 3
Selected applications of gel electrophoresis

Enzyme/source	Comments	References
Thioredoxin reductase Calf liver	Discontinuous native PAGE and SDS–PAGE. Two subunits (M_r=57 kDa). Coomassie staining (protein) and activity (DTNB reduction by NADPH, yellow product).	[167]
Glutathione reductase *Pisum sativum*	Native two-dimensional PAGE and gradient SDS–PAGE. Two subunits (M_r=55 kDa), five isoforms (p*I*s 4.1 to 4.9). Silver staining (protein) and MTT/DCPIP for activity	[161]
Biliverdin reductase Human liver	Single and two-dimensional PAGE, SDS–PAGE. Monomeric (M_r=40.7 kDa). Four isoforms (p*I*s 5.55 to 6.03). Protein staining with Coomassie Blue	[210]
Homoserine dehydrogenase *Daucus carota*	Native PAGE and SDS–PAGE. Subunit (M_r=85 kDa). Multiple forms (trimers, tetramers, hexamers and octamers). Silver staining for protein and NBT/PMS for enzyme activity	[123]
Alcohol dehydrogenase *Thermoanaerobacter ethanolicus*	Discontinuous native PAGE and SDS–PAGE. M_r=40 kDa for the subunit. The native enzyme is tetrameric. Protein staining using Coomassie Brilliant Blue	[195]
D-Xylose dehydrogenase Pig liver	Native PAGE and SDS–PAGE. Two subunits (M_r=32,1 kDa). Staining with Coomassie Blue for protein and with NBT/PMS for enzyme activity in the native gel	[101]
Glutathione reductase Mouse liver	Discontinuous PAGE disc and urea–SDS–PAGE. Two subunits (M_r=55 kDa). Coomassie staining for protein and quenching of yellow fluorescence of NADPH at 260 nm for enzyme activity	[55]
Glycerol dehydrogenase *Aspergillus nidulans*	Gradient SDS–PAGE and IEF. Monomeric (M_r=38 kDa); p*I*=5.1	[25]
Morphine dehydrogenase *Pseudomonas putida M-10*	Native PAGE, SDS–PAGE. Monomeric (M_r=31 kDa); p*I*=4.2. Coomassie Blue staining for protein and NBT/PMS for activity localization in native gels.	[66]
Isocitrate dehydrogenase *Nicotiana tabacum*	Native discontinuous PAGE and SDS–PAGE. Two subunits (M_r=48.5 kDa) and two isoenzymes. Protein revealed with Coomassie and enzyme activity with NBT/PMS.	[26]
Glutamate dehydrogenase *Laccaria laccata*	Uniform and gradient PAGE and SDS–PAGE. Hexameric (subunit M_r=47 kDa). Activity staining coupling the system NBT/PM and Coomassie Blue for protein bands	[211]
Lysine-ketoglutarate reductase *Zea mays*	Discontinous PAGE and SDS–PAGE. Monomeric (M_r=140 kDa). Silver protein staining and activity revealed by the quenching of yellow fluorescence of NADPH at 460 nm.	[158]
Glutathione reductase Eastern white pine needles	Native PAGE and gradient SDS–PAGE. Two different subunits (M_r=53 and 57 kDa) and two dimeric isoforms. Silver staining for protein and MTT/DCPIP for activity	[162]
3-Deoxyglucosone reductase Monkey kidney	Native PAGE, gradient SDS–PAGE and IEF. Multiple forms: homodimers (M_r=39 kDa, p*I*=7,9) and monomeric forms (M_r=38 kDa, p*I*=6.0 and M_r=34 kDa, p*I*=5.6). Activity staining with Meldola Blue/INTC	[164]

protein–wall interactions to separate proteins at femtogram levels [137], are available from Applied Biosystems, BioRad, Supelco, etc. and gel-filled capillaries are available from Beckman Instruments, Applied Biosystems and J&W Scientific. CE equipment is supplied by more than a half-dozen manufacturers.

The growing number of papers published every year in different journals is evidence of the burgeoning interest in this technique. The recently launched Journal of Capillary Electrophoresis is devoted to essential information on the application of CE techniques. A speculative look towards future developments suggests that many exciting innovations in the technique lie ahead and will contribute to the successful separation of complex protein mixtures in the years to come. As far as we know, the use of CE in analysis of $NADP^+$-dependent enzymes remains limited. In 1994, Guttman and Nolan [138] compared the separation of many proteins by SDS–PAGE in slab gels and SDS–PAGE gel-filled capillaries. The behaviour of GSSGRase was different in both systems: subunit M_r values were 62.2 kDa and 54 kDa for capillary and slab gel systems, respectively, both differing from those previously published (M_r 52 kDa) for this enzyme [139].

4.4. Localization of protein bands in gels

4.4.1. Staining for protein

The protein bands are localized after running the gels by Coomassie [140–142] and silver stains [143,144], widely used with many $NADP^+$-dependent enzymes. Silver staining is about 100-fold more sensitive than staining with Coomassie Blue, although the differential staining obtained for different proteins with silver staining is an important drawback. The relationship between the density of silver staining and the protein concentration is characteristic for each protein [145] and, thus, pure enzyme should be included in each gel to quantitate a particular protein. Coomassie staining yields the same colour intensity for different proteins and is usually recommended for densitometric quantitation, while silver staining is useful for detecting minor contaminants. Several methods have been developed to reduce the excessive background of gels [146,147] and to improve protein quantitation [148].

4.4.2. Staining for enzymatic activity

Assignment of individual bands is usually carried out by in situ staining for specific enzyme activities. Several methods have been developed to detect activities in gels and a number of books and review articles have been published [102,149,150]. Electrophoresis under denaturing conditions (SDS-PAGE) yields better resolution than other gel electrophoretic modes, but enzyme activity is irreversibly lost in most cases. Thus, non-denaturing conditions are highly recommended in all steps of the electrophoretic process to localize enzyme activity bands. Renaturation of proteins, to recover enzymatic activity after SDS-PAGE by removing the detergent, has been reported for monomeric and homodimeric enzymes [151–153]. For many dehydrogenases, this method allows only a partial restoration of activity, enough to perform a positive stain [154]. Electrophoresis of many dehydrogenases under native conditions requires protection of their thiol residues; thus, either pre-electrophoresis or polymerization with riboflavin and light, instead of peroxides, is recommended [97,155]. The rehydratable gels used in IEF can be also used for this purpose.

Two methods are generally used for detecting $NADP^+$-dependent enzymatic activity bands in gels. The first is based on reduction of tetrazolium dyes by NADPH, to produce insoluble coloured formazans [104,156,157]. In the second, activity bands are directly visualized in the gels under ultraviolet light [105,149]. Tetrazolium salts are reduced by NADPH only to a small extent. However, in the presence of enzymes with diaphorase activity, NADPH electrons are quantitatively transferred to the tetrazolium salt [159]. Electrons can also be non-enzymatically transferred via phenazine methosulfate (PMS) [160], 2,6-dichlorophenolindophenol (DCIP) [161,162] or Meldola Blue (MB) [163] with full effectiveness. Insoluble formazans are deeply coloured, monoformazans from yellow to red and diformazans from blue to black. Different commercial tetrazolium salts can give formazans of different colour intensity; thus, previous calibration is required for quantitation. Monoformazans yield a more sensitive stain than diformazans. With respect to the NADPH absorbance at 340 nm, the sensitivity can be easily increased by factors ranging between three and five-fold. Activity staining can be carried out in the oxidative direction

(dehydrogenase activity, positive stain) [149] or in the reductive direction (negative stain) [160–164]. These methods are sensitive and can be carried out with a single incubation mixture yielding sufficient stable stain; old gels can display excessive background due to the effects of light and oxygen. Unspecific dehydrogenase activities leading to NADPH formation should be controlled in parallel experiments carried out in the absence of substrates.

The activity stain using UV light is based on the yellow fluorescence of NADPH, non-fluorescent in the oxidized form, which can be visualized by means of transilluminators. The activity stain appears either as dark bands in a yellow fluorescent background (reductase negative stain) or as fluorescent bands on a dark background (dehydrogenase positive stain), according to the oxidized and reduced forms of the coenzyme, respectively [105,165]. The ability to stain in both directions could be used with some advantages, for instance when the activity in one direction is greater than in the opposite direction. False-positive bands can be detected after preincubation with the pyridine nucleotide in the absence of the other specific substrate. For an adequate photographic record of gels, careful control of the incubation time must be observed, due to continuous diffusion of substrates. If a coloured product is produced by the enzymatic reaction, the activity can be directly visualized in the gel. Thus, Martínez-Galisteo et al. [167] have reported an in situ assay for thioredoxin reductase activity [168], by visualizing the yellow band corresponding to the product of the reduction of the substrate 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by NADPH.

Analytical gel electrophoresis under its different modalities, has been reported in almost 95% of the reviewed articles published since 1990. Among them, SDS–PAGE was the most used (ca. 92%), followed by native PAGE (ca. 47%), while about 14% of protocols used IEF and only ca. 8% used preparative gel electrophoresis. Other electrophoretic techniques were described in very few papers (ca. 3%).

5. Activity assay methods

Up to now several hundred enzymatic activity assay methods have been reported for $NADP^+$-linked oxidoreductases, and these are classified as continuous or discontinuous methods. In a continuous method, direct observations are made within the assay mixture while the reaction is going on. In a discontinuous method, the reaction is followed by withdrawing samples at various times, and the assayed parameters are determined after stopping the enzyme reaction. HPLC-based assay methods are, thus, discontinuous.

5.1. Continuous assay methods

The substrates or products of many enzyme reactions may absorb light, either visible or in the UV region of the spectrum. Since it is unlikely that they would have identical spectra, it is possible to find a wavelength at which the conversion of a substrate into a product is reflected by an absorbance change to measure the progress of the enzymatic reaction, which is the basis of continuous spectrophotometric assay methods. For most $NADP^+$-linked oxidoreductases, activity is followed by measuring the absorbance change at 340 nm, a wavelength at which only the reduced pyridine nucleotides absorb ($\epsilon_{1\ mM,\ 1\ cm}$ = 6.22). Several hundred references could be quoted in relation to spectrophotometric-based assay methods for these enzymes.

For reactions in which either substrate or product absorb near 340 nm, it could be necessary to follow the reaction rate at wavelengths within the visible region of the spectrum. To achieve this, the enzymatic reaction must be coupled with a redox dye system, such as the tetrazolium salts used for activity staining in gels. An assay for $NADP^+$–glycerol dehydrogenase coupling of the enzymatic reaction with the system NBT/PMS [169,170] was reported by Schuurink et al. [25]. The reaction rate was followed by monitoring the absorbance at 578 nm, the absorption maximum of the soluble formazan produced. Another alternative to avoid interferences from the assay mixture would be to follow the reaction kinetics by fluorescence. Reduced pyridine nucleotides show a blue fluorescence (λ_{em}=440 nm), while the oxidized forms do not fluoresce [171,172] and this can be used to assay the activity of these enzymes [173]. The fluorimetric assay is at least 100-fold more sensitive than the photometric assay.

Spectrophotometric assays are usually preferred to any other type due to their facility and sensitivity,

which is enough for most applications. Spectrophotometers are basic laboratory instruments with thermostatted cuvette exchangers and computer software control, allowing the recording of kinetic data at any wavelength in the UV–Vis regions simultaneously for many samples. Whenever either the products or the substrates cannot be continuously followed in the initial reaction mixture, a discontinuous assay method is necessary to monitor the reaction rate.

5.2. Discontinuous assay methods

In the last few years, the power and versatility of HPLC has increased greatly due to computerization of autosamplers, pumps, detectors, data capture and peak analysis by low-cost personal computers [174–178]. New advances in robotic handling of samples, special devices to perform pre- and post-column derivatizations, premixing operations in autosamplers and new column technologies [179–182] have made HPLC a very powerful separation technique to assay enzymic activities in a high number of samples which can be processed overnight. Different strategies to design HPLC-based assays for many enzymes have been described by Rossomando [183–185]. Recent applications of the technique have been reviewed by Lambeth and Muhonen [186]. The popularity of HPLC-based enzyme assays continues to grow and, to date, the number of published methods has risen to several hundred.

Some enzymatic reactions occur at a slow rate and, thus, require long times and sensitive detection to follow the reaction. In these cases, HPLC could be the method of choice to carry out the assay. The HPLC-based methods might prove useful to assay enzyme activities in crude extracts where many additional enzymes are present and, therefore, unclear results are obtained by conventional methods. In any HPLC assay, kinetic data are obtained from the chromatogram and, hence, correct selection of the type of column, mobile phase, flow-rate and detector can be made to achieve a rapid separation and sensitive quantitation of the reaction components. To obtain initial rates, assay conditions such as pH, composition of the assay mixture, enzyme concentration, temperature and incubation time must be optimized [187,188]. An appropriate procedure to efficiently stop the enzymatic reaction is also necessary. This can be accomplished in several ways, such as by the addition of strong acids, bases or inhibitors, boiling, chilling in ice, extraction with organic solvents or direct injection of the assay mixture onto the column [183–185]. Sometimes, addition of an internal standard can be useful to assess the recovery of substrate or product through the different steps of the activity assay [189–193].

In the recent literature, HPLC-based methods to assay $NADP^+$-linked oxidoreductases have been reported in about 11% of papers examined. Some relevant features of the assays for representative enzymes are summarized in Table 4.

6. Conclusions

For the purification of pyridine nucleotide-dependent oxidoreductases, it is essential to design a purification strategy maximizing purity, yield and economy, taking full advantage of the many separation technologies currently available.

The high growth rate of chromatographic techniques in the last two decades has somewhat slowed down in recent years and it is generally accepted that more modest advances can be expected in the years to come. As an example, in spite of their recognized drawbacks, soft gels have been used in at least 65% of protocols reviewed. Nevertheless, in our opinion, chromatography may have a brilliant future for protein separation. Thus, a main goal could be to design highly specific polymeric supports for the tailor-made isolation of an individual enzyme using molecular imprinting [213,214]. This technique has been successfully applied to the isolation of small molecules and its implementation for protein purification remains a challenge for the future. In addition, all chromatographic modes used for protein purification may be speeded up by using the new perfusion chromatography methodology, useful for micropreparative separations of proteins with almost quantitative recovery of enzymic activity, operating at low to medium pressures [38–40].

Over the past six years, CE has captured the interest of many biochemists as a new tool for solving many separation problems. Further advances in capillary coating and detection systems are needed to make this methodology a powerful technique for routine work. We expect that capillary protein se-

Table 4
Some features of HPLC-based activity assays for $NADP^+$-dependent oxidoreductases

Enzyme	Teminating step	Internal standard	Detection	Column type	Mobile phase	Flow-rate ml/min	Reference
Salicylhydroxamic acid reductase	NaOH	Benzamide	UV 254 nm	C_{18} (150×4.6 mm I.D.) (M&S PaK)	Acetonitrile–0.1 *M* KH_2PO_4, (2:8, v/v)	0.7	[190]
α,β, Ketoalkene double bond reductase	Ice	*p*-Hydroxybiphenyl	UV 254 nm	Lichrosorb RP18 (125×4 mm I.D.) Merck	Acetonitrile–water (2:3, v/v)	0.5	[189]
Glutamate dehydrogenase	HCl		Fluorescence λ_{ex}=338 nm, λ_{em}=425 nm	μBondapack C_{18} (150×3.9 mm I.D.) Waters	20 m*M* sodium phosphate, pH 6.5; 22% methanol and 2% tetrahydrofurane (v/v)	1–1.5	[208]
2′-Hydroxydaidzein oxidoreductase	Ethyl acetate extraction		UV 278 nm Fluorescence	Lichrosorb RP18 (60×4 mm I.D.) Merck	Methanol–water 40:60 (v/v)	1.0	[166]
Carbonyl reductase	HCl	3-acetyl-7-dimethyl aminocoumarin	λ_{ex}=315 nm, λ_{em}=375 nm	Chiralcel OD Daicel, Japan	*n*-Hexane–2-propanol (93:7, v/v)	1.2	[193]
Lysine-ketoglutarate reductase	Perchloric acid	β-guanidino-propionic acid	Electrochemical	Ultrasphere XL ODS (70×4,6 mm I.D.) Beckman	gradient elution, 0.1 *M* sodium acetate, pH 6.7 and methanol	1.6	[191]
Dihydropyrimidine dehydrogenase	Trichloro-acetic acid		UV 275 nm	Hypersil ODS2 Shandon Southern	20 m*M* KH_2PO_4, pH 5.6–methanol (94:6, v/v)	1.0	[209]
5α-Steroid reductase	Diethyl ether extraction	Methyl lithocholate	UV 215 nm	Hi-Chrom-Ciano (250×5 mm I.D.) Reading UK	Hexane–isopropanol (9:1, v/v)	1.0	[192]

quencing, capillary tryptic mapping and capillary amino acid analysis will become routine analytical techniques in the near future.

The goals of new method development are to save time and sample. The increase in biotechnological processes and the need for highly purified active proteins will greatly promote some of these techniques in the near future. The integration of new methodologies in analytical and preparative separation schemes will be a challenge in the coming years.

7. List of abbreviations

AC	Affinity chromatography
2′,5′-ADP	Adenosine-2′,5′-diphosphate
BAC	Bisacrylylcystamine
CE	Capillary electrophoresis
CF	Chromatofocusing
CM	Carboxymethyl
CTAB	Cetyltrimethylammonium bromide
C8ATPR	8-(6-Aminohexyl)-amino-2′-phosphoadenosine diphosphoribose
DEAE	Di-ethylaminoethyl
DMIRase	7,2′-Dihydroxy-4′,5′-methylene-dioxyisoflavone oxidoreductase
DCIP	2,6-Dichlorophenolindophenol
DTNB	5,5′-Dithio-bis-(2-nitrobenzoic acid)
EBAC	Expanded bed affinity chromatography
FPLC	Fast protein liquid chromatography
GSSGRase	Glutathione reductase
GSSG	Oxidized glutathione
G6PDH	Glucose-6-phosphate dehydrogenase
HEPES	Hydroxyethylpiperazine ethanesulfonic acid
HIC	Hydrophobic interaction chromatography
HPLAC	High-performance liquid affinity chromatography
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
INT	*p*-Iodonitrotetrazolium chloride
LC	Low-pressure liquid chromatography
MB	Meldola Blue (8-methylamino-2,3-benzophenoxazine)
MOPS	3-(N-Morpholine) propane sulfonic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced $NADP^+$
NBT	2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′diphenylene) tetrazolium chloride
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethyleneglycol
PEEK	Polyetheretherketone
PEI	Polyethyleneimine
PERCAS	Perfluorocarbons emulsion reactor for continuous affinity separations
PIPES	Piperazine-N,N′-bis(2 -ethane)sulfonic acid
PMS	Phenazine methosulfate.
QA	Quaternary ammonium
RPC	Reversed-phase chromatography
SEC	Size-exclusion chromatography
SDS	Sodium dodecyl sulfate
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SP	Sulfopropyl
TEMED	N,N,N′,N′-Tetraethyl-methylenediamine

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