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Review

Fluorogenic substrates for hydrolase detection following electrophoresis

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Abstract

Fluorogenic substrates provide a highly sensitive and in many instances specific method to detect hydrolytic enzymes after electrophoretic separations. Factors affecting sensitivity and specificity of the title substrates are generally discussed. Techniques of fluorogenic substrate application after polyacrylamide, agarose or starch gel electrophoresis, cellulose acetate electrophoresis and isoelectric focusing are treated in detail. Examples of the detection of esterases, glycosidases and peptidases using fluorogenic substrates such as naphthol, naphthylamine, 4-methylumbelliferone and 4-methyl-7-aminocoumarin derivatives are given. Applications to clinical, biological and food chemistry are outlined.

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1. Introduction

Fluorogenic substrates are generally much more sensitive than chromogenic in enzyme assays [1]. General aspects and first examples of application may be found in monographs by Udenfriend [2] and Elevitch [3]. Moss et al. [4] were the first to detect a hydrolase, alkaline phosphatase (EC numbers of hydrolases are given in Tables 2–4), after starch gel electrophoresis by its action on 1- and 2-naphthyl phosphate which resulted in the liberation of the fluorescent naphthols. The relationship between isoenzyme patterns and chronic or acute diseases on the one hand and the possibility of analysing very small samples of biological material on the other stimulated the development of other fluorogenic substrates, the inclusion of other electrophoretic techniques and their introduction into clinical laboratories. Similar studies on enzymes from other classes, in particular those on the isoenzymes of L-lactate dehydrogenase (EC 1.1.1.27) [3,5–7] and creatine kinase (EC 3.7.3.2) [7–12] with the fluorogenic cosubstrate NAD, considerably influenced these developments. The possibility of determining the enzymes in small biological samples with the same sensitive substrate as used to detect the enzymes after electrophoresis offered additional advantages.

This review deals with fluorogenic substrates that are used directly by the enzyme studied yielding fluorescent products. Not treated are fluorogenic substrates or cosubstrates used after the application of auxiliary enzymes in indicator reactions or fluorogenic reagents used to detect enzyme products. Hydrolases have been selected because in this class various fluorogenic substrates have been used for a large number of enzymes. In the remaining five classes of enzymes, fluorogenic substrates and/or cosubstrates have only been used for the detection of the above-mentioned L-lactate dehydrogenase and creatine kinase isoenzymes, γ-glutamyltransferase (EC 2.3.2.2) [13,14], N-acetyllactosamine synthase (EC 2.4.1.90) [15] and carbonate dehydratase (EC 4.2.1.1) [16,17]. Electrophoretic techniques considered are those separating the enzymes according to charge and/or molecular mass such as paper electrophoresis, cellulose acetate electrophoresis (CAE), starch gel electrophoresis (SGE), agarose gel electrophoresis (AGE), native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS) PAGE and isoelectric focusing (IEF) in agarose and polyacrylamide gel.

2. General aspects

2.1. Principle of fluorogenesis

Most of the fluorogenic substrates used for the detection of hydrolases after electrophoresis contain a leaving group at the bond attacked by the enzyme which is not fluorescent when bound and exhibits fluorescence when liberated by the enzyme. Table 1 shows that only a limited number of such leaving groups have been used. The number of the respective fluorophores is even smaller, mainly naphthalene (Fig. 1, **1**, **2**, **4** and **7**) and coumarin (Fig. 1, **6** and **8**) derivatives have been used, with the coumarins predominating (see also Tables 2–4).

A different principle, quenching, has recently been employed in two substrates. Self-quenched acetamidofluorescein–bovine serum albumin (AF–BSA) was used to detect cathepsin D and other endopeptidases after electrophoresis [18] (see also Table 4). Although AF–BSA contained about 25 randomly distributed fluorescein groups per molecule, it was only very weakly fluores-

Table 1
Leaving groups in fluorogenic substrates for hydrolase detection

Leaving group	Fluorescent compound	Formula[a]	λ_{ex}[b]	λ_{em}[b]
1-Naphthoxy	1-Naphthol	**1**	335	455
2-Naphthoxy	2-Naphthol	**2**	350	425
N-Methylindoxyl	1-Methyl-3-oxyindole	**3**: R=CH_3		500
N-Methoxyindoxyl	1-Methoxy-3-oxyindole	**3**: R=OCH_3		510
Naphthol AS	3-Hydroxy-2-naphthoic acid anilide	**4**: R^1=R^2=R^3=H		
Naphthol AS-BI	6-Bromo-2-hydroxy--3-naphthoic acid 2′-methoxyanilide	**4**: R^1=Br, R^2=OCH_3, R^3 = H	405	505
Naphthol AS-D	3-Hydroxy-2-naphthoic acid 2′-methylanilide	**4**: R^1=R^3=H, R^2=CH_3		
Naphthol AS-MX	3-Hydroxy-2-naphthoic acid 2′, 4′-dimethylanilide	**4**: R^1=H, R^2=R^3=CH_3	405	505
Naphthol AS-TR	3-Hydroxy-2-naphthoic acid 4′-chloro-2′-methylanilide	**4**: R^1=H, R^2=CH_3, R^3=Cl		
Fluorescein	Fluorescein	**5**	495	570
MU	4-Methylumbelliferone	**6**: R=CH_3	360	450
TFMU	4-Trifluoromethyl umbelliferone	**6**: R=CF_3	352	445
NA	2-Naphthylamine	**7**: R=H	340	425
MNA	4-Methoxy-2-naphthylamine	**7**: R=OCH_3	340	425
MCA	4-Methyl-7-aminocoumarin	**8**: R=CH_3	380	460
TFMCA	4-Trifluoromethyl--7-aminocoumarin	**8**: R=CF_3	400	505

[a] Structures of fluorescent compounds are shown in Fig. 1.
[b] λ_{ex} = Excitation wavelength (nm); λ_{em} = emission wavelength (nm); data from the literature for maxima or wavelengths most frequently used (also wavelengths differing by a few nm have sometimes been used).

cent. If many molecules of a fluorophore are covalently attached to a protein, electronic interaction between neighbouring fluorophores leads to fluorescence quenching. Proteolysis increases the mean distance between the fluorophores, so that quenching is relieved and products are obtained which are more intensely fluorescent than the intact substrate (λ_{ex} = 492 nm, λ_{em} = 517 nm).

Similarly, the intramolecularly quenched substrate Dans–D-Ala–Gly–Phe(NO_2)–Gly was used to detect neprilysin after SDS-PAGE [19] (Table 4). Cleavage of the glycyl-*p*-nitrophenylalanine–peptide bond separates the quenching nitrophenyl residue from the fluorescent dansylpeptide (λ_{ex} = 342 nm, λ_{em} = 562 nm).

2.2. Sensitivity

To evaluate the feasibility of a fluorogenic substrate, its specificity and sensitivity must be considered. The sensitivity depends on a number of factors.

2.2.1. Fluorescence intensity

First, the detection limit of the fluorescent product formed, which is related to the wavelength of excitation and emission used, to the molar absorptivity at the excitation wavelength and to the quantum yield of fluorescence is of importance. Excitation of fluorescent products by a UV lamp for scanning should be done at least 50 nm below the emission wavelength when quantification of enzymatic activity is intended.

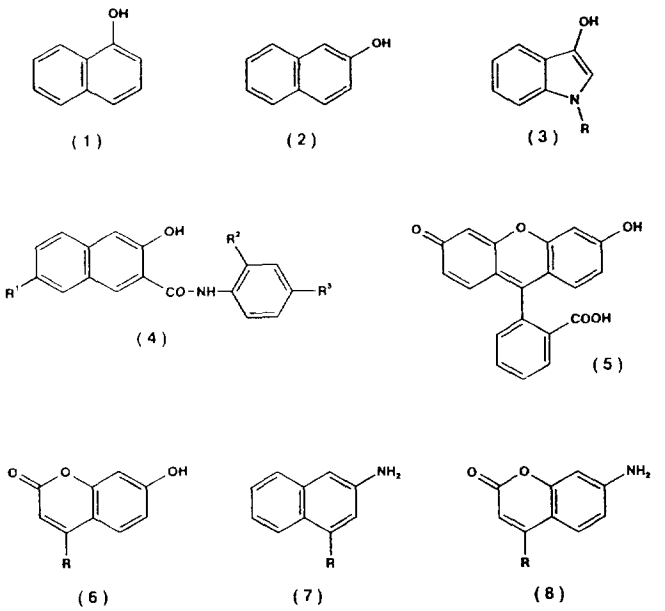

Fig. 1. Structures of fluorescent compounds released from fluorogenic hydrolase substrates. Names and substituents (R) are given in Table 1.

Otherwise, light from the excitation source may influence the result. This implies that the fluorophore might be excited at a wavelength lower than that of maximum absorbance which, of course, lowers the sensitivity. The emission spectrum should also be adapted for the kind of evaluation used, visual location, photographing or measuring with a fluorimeter or densitometer, which can be done only by selecting the suitable fluorophore. This was stated by Moss et al. [4], who recommended 1-naphthyl phosphate to visualize alkaline phosphatase, as the blue fluorescence of 1-naphthol is more apparent to the eye than that of 2-naphthol, and 2-naphthyl phosphate to measure its activity. Molar absorptivity is a property which may depend on solvent or pH, as also may quantum yield. However, selection is limited, as enzymatic reactions proceed in aqueous media at a definite pH. The pH may be adjusted to that of maximum fluorescence after the reaction has proceeded, but this requires a further step in the detection procedure which may result in band broadening. Again, a better choice is the selection of a more suitable substrate which contains a leaving group that exhibits higher fluorescence. In peptidase detection, 4-trifluoromethylcoumarinyl-7-amide (TFMCA) substrates are more sensitive than 4-methoxy-2-naphthylamide (MNA) substrates, ten times in terms of molar fluorescence intensity [20], and also more sensitive than 4-methylcoumarinyl-7-amide (MCA) substrates [21]. However, the sensitivity of MNA derivatives can be enhanced by reacting with 5-nitrosalicylaldehyde. A twofold effect is obtained by the reaction. First, the fluorescence is more intense and second, the single bands are much sharper owing to the relative insolubility of the product formed [21]. To detect phosphatase, naphthol AS-MX phosphate is 2.8–24 times more sensitive than 1-naphthyl phosphate, depending on the excitation and emission wavelengths used, and naphthol AS-MX has a 43% higher apparent molar fluorescence than naphthol AS-BI [22]. 4-Methylumbelliferone emits greater fluorescence than do naphthol AS and its derivatives; the ranking of the latter according to decreasing fluorescence is naphthol AS, naphthol AS-TR and naphthol AS-D, naphthol AS-BI [23].

2.2.2. Reactivity

The second factor of importance is the reactivity of the substrate, which depends on the affinity of the substrate to the enzyme, K_m, and the observed rate constant, k_{cat}. Reactivity varies between different fluorogenic substrates for the same enzyme. For example, k_{cat}/K_m values of 7.6×10^5 l mol^{-1}s^{-1} for Z–Gly–Gly–Arg–TFMCA (Z = benzyloxycarbonyl) [24], 1.9×10^5 l mol^{-1}s^{-1} for Z–Gly–Gly–Arg–MCA [24], 4.5×10^3 l mol^{-1}s^{-1} for Z–Arg–MCA [25] and 3.8×10^3 l mol^{-1}s^{-1} for Bz–Arg–MCA (Bz = N^2-benzoyl) [25] have been reported for tryspin, and values of 1.7×10^3 l mol^{-1}s^{-1} (Ala–Ala–Phe–MCA) and 78 l mol^{-1}s^{-1} (Glt–Phe–MCA) (Glt = glutanyl) for chymotryspin [26]. At least within these examples, reactivity seems to be related mainly to the peptide moiety of the substrates.

2.2.3. Solubility

Further, the solubility of the substrates and the fluorescent leaving groups are of importance. The solubility of the substrate should be high and diffusion into the gel fast when direct staining or overlay techniques with filter-paper or agar(ose) gel are used to detect the enzymes. On the other hand, the solubility of the product should be low and its diffusion slow, as otherwise band broadening occurs [23].

A further aspect that favours coumarin derivatives is the higher toxicity of the MNA derivatives [27].

2.3. Specificity

The substrates that have been used to detect esterases are largely unspecific, as demonstrated by the examples listed in Table 2 (carboxylic ester hydrolases, alkaline and acid phosphatase). In their study on human tissue esterases with N-methylindoxyl acetate and various 4-methylumbelliferyl (MU) and fluorescein esters, Coates et al. [17] were able to classify the isoenzymes according to their different substrate preferences. At least thirteen different esterase isoenzymes or sets of isoenzymes have been found in human tissues which differ in their substrate specificity, electrophoretic mobility and tissue distribution.

The substrates used to detect the glycosidases are highly specific substrates owing to the carbohydrate moiety bound to the fluorogenic leaving group and the configuration of that bond, for both of which the glycosidases are specific. The MU β-lactoside may be regarded as an exception, as it is also cleaved, to a different extent, by the two non-specific *endo*-1,4-β-glucanases present in the cellulose- and xylan-degrading enzyme complex [28,29].

In order to detect peptidases after electrophoresis, highly specific or less specific substrates may be needed, depending on the problem. When biological samples are screened for peptidases with unknown specificity, the best substrate is that which is attacked by as many peptidases as possible. In such cases endopeptidase activity can be detected with AF-BSA, a substrate that fluoresces if peptide bonds are split, independently of the amino acids between which cleavage occurs [18]. Similarly, D-Val–Leu–Arg–TFMCA can be used as a broad-spectrum substrate to detect serine endopeptidases [30], and substrates such as Z–Phe–Arg–MCA, Suc–Ala–Phe–Lys–MCA (Suc = succinyl) or Leu–Val–Tyr–MCA to detect cysteine endopeptidases [31] (see also Table 4).

In most instances, however, fluorogenic substrates are used to detect enzymes specifically in biological samples with low activity containing several enzymes. Dipeptidyl-peptidase III was specifically detected with Arg–Arg–NA (NA = 2-naphthylamide) and Leu–Ala–NA at pH 9.0 after electrophoretic separation of lens exopeptidases [32]. Ala–Ala–Phe–MCA and Z–Gly–Gly–Arg–TFMCA, impregnated into membranes, selectively detect chymotryptic and tryptic activity, respectively, in human duodenal juice [33]. However, it should be emphasized that the reported specificity is valid only for the system studied. It is recommended to check the substrate used for its specificity under the actual experimental conditions, in particular with regard to other enzymes present. One way to do so is to suppress the activity studied by a specific inhibitor. If the substrate is specific for that

activity, no activity should be detected thereafter. The specificity of substrates for a given enzyme may also be enhanced by the addition of inhibitors against accompanying enzymes. Thus, the broad-spectrum serine endopeptidase substrate D-Val–Leu–Arg–TFMCA, along with soybean trypsin inhibitor and buffered at pH 8.2, was impregnated into membranes and used to study tissue kallikrein [34]. Similarly, Bz–Val–Lys–Lys–Arg–TFMCA in the presence of aprotinin, the bovine pancreatic trypsin inhibitor, was used for the study of cathepsin B [34].

3. Application techniques

The procedures used to detect enzymatic activity with fluorogenic substrates after electrophoresis can be arranged into three groups with regard to the basic technique: (1) elution followed by assay in solution (the supporting material is cut into pieces, the pieces are separately eluted and the eluates are tested for the enzyme in test-tubes using the fluorogenic substrate), (2) direct staining of the complete gel [application of substrate in solution in many variations from immersing to spraying, the simplest and most frequently used procedures, after starch gel electrophoreins (SGE), agarose gel electrophoresis (AGE), PAGE, IEF and cellulose acetate electrophoresis (CAE)], and (3) indirect staining of the complete gel (overlay techniques with the substrate applied on a support that is placed on the electrophoresis gel). Almost all detection procedures may be used following any of the electrophoretic techniques, but some combinations predominate (direct staining after SGE, CAE, PAGE, SDS-PAGE or IEF; filter-paper overlay after SGE; agarose gel overlay after CAE; cellulose membrane overlay after IEF). Owing to different conditions existing in the supporting material after various electrophoreses, detection procedures have been adapted to separation techniques.

3.1. Elution technique

The protocol of an elution followed by an assay in solution is very similar to that of a common fluorimetric enzyme assay in test-tubes. At the end of the electrophoretic run, either the strips of filter-paper [35] or slab gels (after IEF [36]), or the gel rods (after PAGE [36]) were cut into several equal pieces, or the protein bands stained with Ponceau S in a small edge were cut out of a strip (after CAE [37]). The pieces were put into the reaction vessels containing the assay mixture with the fluorogenic substrate. After incubation at 25 or 37°C for about 30 min [35] or 16 h [36], the reaction was terminated by addition of a usual stopping reagent, and the fluorescence of the hydrolysis products, 4-methylumbelliferone [35,37] or 4-methyl-7-aminocoumarin [36], was determined using a spectrofluorimeter. McGuire and DeMartino [38] homogenized each gel slice (2 mm slices from rod gels and 3 mm slices from slab gels, after PAGE) in 200 μl of 50 m*M* Tris–HCl buffer (pH 8.0) prior to the determination of 'macropain' (EC 3.4.99.46) activity by measuring 4-methoxy-2-naphthylamine produced from Z–Val–Leu–Arg–MNA in the usual way. Even in 1992, in addition to the cellulose membrane overlay technique, Nwagwu et al. [39] still used this technique in a modification. They blotted chymotryptic-like activity of PAGE gel on to DEAE paper (4°C, 2.5 h), cut the DEAE paper into 5 mm strips, transferred the strips into tubes containing 2 ml of elution buffer (pH 7.0), stirred the tubes vigorously and incubated them on ice for 1 h followed by centrifugation. The supernatant was examined by proteinase assay.

The procedures are certainly appropriate to identify and characterize a few enzymes with different or identical activities in one sample, but not to compare several samples and/or a larger number of isoenzymes in the individual sample. The latter problems are solved better by direct staining.

3.2. Direct staining

Application of a fluorogenic substrate in solution was first used by Johnson [22] to detect alkaline serum phosphatase in starch gels. In order to optimize the procedure, Johnson studied a number of factors that influence staining, such as the kind of substrate and of buffer

components, pH, incubation temperature and activating (e.g., magnesium ions) or inhibiting (e.g., EDTA) compounds. Comparison of the substrates 1-naphthyl phosphate, naphthol AS-MX phosphate and naphthol AS-BI phosphate showed naphthol AS-MX phosphate to be the most sensitive ($\lambda_{ex} = 405$, $\lambda_{em} = 505$ nm). The best results were obtained with 2 m*M* substrate solution. The reaction was found to be influenced also by the buffer system. Johnson [22] introduced 2 *M* 2-amino-2-methyl-1-propanol buffer (pH 9.8), which was later frequently used also by other groups. As the enzymatic reaction proceeds best at pH 9.8, the buffer with its p*K* of 9.6 can best resist the acidifying action of the gel buffer. The way in which the gel is incubated with the substrate is different from procedures used later more frequently. In a glass tray, the buffered substrate solution is poured over the SGE gel, covered with Saran Wrap and placed in a 37°C bacteriological incubator for 2 h. The gel is removed and viewed under long-wavelength UV radiation.

Of course, the staining conditions have to be adapted not only to the electrophoretic conditions but also primarily to the enzymes studied. For example, to locate isoenzymes of human erythrocyte acid phosphatase, the SGE gels were incubated at 37°C in a substrate–buffer solution (1 m*M* MU phosphate in 0.1 *M* acetate buffer, pH 5.5). The formation of the fluorescent product, 4-methylumbelliferone, was monitored continuously under UV radiation [40]. To identify carboxylesterases, Coates et al. [17] incubated SGE gels at 37°C in 0.01% substrate in 0.1 *M* phosphate buffer (pH 6.5). The substrates, fluorescein diacetate, N-methylindoxyl acetate and esters of 4-methylumbelliferone, were dissolved in a few millilitres of acetone before mixing with the buffer. To the longer chain esters also a few drops of 1% Triton X-100 were added to aid solution. Coates et al. also varied the procedure depending on the substrate. Short-chain esters from acetate to butyrate were applied by filter-paper overlay because only a short period of incubation (minutes) is necessary. The longer chain esters were used in fluid stains because the gels were incubated for several hours. With regard to different pH values of the electrophoresis gels, acid and neutral gels generally cause less problems than alkaline gels. For that reason, Coates et al. [17] recommended presoaking alkaline gels in 0.5 *M* acetate buffer (pH 5.2) or 0.1 *M* phosphate buffer (pH 6.5) for 30 min to minimize background fluorescence due to alkaline hydrolysis and liberation of free 4-methylumbelliferone.

Application of fluorogenic substrates in solution following PAGE is similar to that after SGE. In most instances, the gels are incubated in substrate solution at 37°C, but also at 20, 45 or 60°C. The incubation time varies between 5–10 min and 1–3 h, depending on the enzyme activity. Rod gels [41–44] generally need longer incubation times than slab gels, as the latter are only 0.5–3 mm thick. Some modifications have been reported for detecting enzymatic activity in rod gels. Friedland et al. [41] placed the gel rods on filter–paper soaked with buffer and sprayed the rods with substrate solution. After staining each gel was placed in a small rectangular quartz cell containing sufficient buffer and scanned in a Turner automatic scanning unit. Chang et al. [42] incubated the gel rods first in substrate solution only for 15 min at 0°C in the dark, then poured off the substrate solution and incubated the rods for further 15–60 min at 37°C. Berg et al. [44] incubated the gel rods first in buffer (15–30 min) and then in substrate solution (60 min). Development of fluorescence was always controlled under a long-wavelength UV lamp and terminated when fluorescent bands were visible. Furthermore, Chang et al. [42] enhanced the fluorescence of liberated 4-methylumbelliferone after the incubation by immersing each gel in 2 ml of 0.12 *M* glycine–0.25 *M* sodium carbonate buffer (pH 10.4) for 2 min before the gels were photographed.

The IEF slab gels are treated with fluorogenic substrate solutions as described above for native PAGE slab gels. One modification only has been reported by Berg et al. [44]. The IEF slab gel was sprayed with substrate solution and incubated at room temperature until fluorescent bands appeared.

After SDS-PAGE, enzymatic activity has only been detected with fluorogenic substrates in solution. The method is not appropriate for all

enzymes as it is necessary that the SDS-treated enzymes can be reactivated by washing procedures for the removal of SDS. Teeri et al. [45] washed the separation gel after electrophoresis once for 15 min with agitation in cold Z-buffer [0.1 *M* sodium phosphate (pH 7.5)–10 m*M* KCl–1 m*M* $MgSO_4$–50 m*M* 2-mercaptoethanol] and twice for 15 min each in Z-buffer at room temperature. Sullivan and Johnson [19] removed the SDS from the gel by washing three times in 10 m*M* Tris–HCl buffer with 0.5% CHAPS and then with the same buffer containing NaCl and urea. Usually washing was done with 2.5% Triton X-100, for 30 min without change [31,46] or for 1 h changing the detergent solution four times [47]. To reveal enzyme bands, the gels were incubated at 37°C (room temperature [45], 4°C [19]) with the appropriate substrate at the corresponding pH with shaking. Fluorescent bands could be seen under UV radiation (350 or 365 nm) after 5–30 min. The results were recorded by photography under UV illumination (details given are as follows: through a yellow filter, Kodak Wratten gelatine filter 2E [31,45], with a Polaroid camera [31], with a Polaroid 55 film [46]). Santana et al. [47] photographed by using a Kodachrome colour film, and the negative was processed to obtain black-and-white prints. Sullivan and Johnson [19] illuminated with a 304-nm transilluminator and photographed with a MP-4 Polaroid camera system equipped with a 65A Wratten gelatine filter.

A further modification of the technique was described by Demetriou and Beattie for the detection of alkaline phosphatase after separation by AGE [48]. Substrate (0.5 m*M* naphthol AS-MX phosphate)–incubation buffer (pH 9.8) was applied as a streak on a glass plate. The agarose film was laid, starting with the sample application end, on the streak so that the substrate–incubation buffer was distributed between the film and glass surface. The film and glass plate were incubated in a glass tray of a waterbath at 37°C for 2 h. The incubator was covered with a black cloth to protect the photosensitive product of the reaction. After the incubation, the agarose film was dried at 80°C for 15 min. After drying, the fluorescent product was stable to light exposure. The film was cut into strips to separate sample lanes, and scans were made with an automatic strip scanner (λ_{ex} = 360 nm, λ_{em} = 505 nm). However, the procedure described for the detection of enzymes after PAGE can also be used after AGE, as has been shown by Nielsen et al. [49] in studies on β-galactosidases.

To detect enzymes after CAE by direct staining, two procedures have also been described. Fritsche and Adams-Park [50] stained the gels for the identification of alkaline phosphatases according to the method reported by Demetriou and Beattie [48]. Another procedure was described by Choy and Davidson [51] and Rattazzi et al. [52] for the deteection of β-glucosidase and arylsulfatase, respectively, with MU derivatives. After the run, the gels were blotted between two pieces of filter-paper to remove any excess of buffer and then immersed in substrate solution [0.5 m*M* MU β-D-glucopyranoside in buffer (pH 5.3) and 15 m*M* MU sulfate in buffer (pH 7.0)] for 60 and 30–60 s, respectively. Again, excess of buffer (with substrate) was removed by blotting between filter-paper. The sheets were incubated in a moist chamber at 37°C for 1–2 h. After incubation, the gels were immersed in a stopping buffer (pH 10.3) for 30 s at 20°C [51], or fixed in formalin for 4 min followed by immersion in buffer (pH 10.0) for 4 min [52]. Fluorescent bands were seen under long-wavelength UV radiation (365 nm).

3.3. Indirect staining

In addition to direct staining, indirect overlay techniques have very frequently been used, in which substrates are dissolved in agar(ose) gel, soaked in filter-paper or impregnated on cellulose or nylon membranes prior to laying these supports on the separation gel.

3.3.1. Agarose gel overlay technique

Following SGE, this technique was described by Martin et al. [53] for acid phosphatase isoenzymes and by Lafferty et al. [32] for some peptidases. The former group mixed the MU phosphate solution with an agar solution cooled to 50°C, poured the mixture onto a glass plate

and placed the electrophoresis gel on the solidified substrate gel. The gels were incubated at 37°C for 10–15 min and monitored under UV radiation. They also used the technique after CAE and discussed its feasibility after PAGE. Lafferty et al. mixed the substrate–buffer solution with the agarose solution at 57°C immediately before pouring it over the surface of the electrophoresis gel. The double gel was incubated at 37°C for 1–4 h until fluorescent bands appeared under UV radiation.

After CAE, agarose gel overlays were also applied by some workers [54–56] as described above [53]. Incubation periods were between 10 and 60 min depending on enzyme activity. Bergerman and Blethen [55] dried the cellulose acetate strips after carefully removing them from agar at room temperature for about 30 min. Thereafter, the fluorescent spots were relatively stable and could be scanned at any time up to 24 h. Alkaline [54,55] and acid [53,56] phosphatases have been detected in this way.

Also 10 years later the agarose gel overlay technique was still being employed, but at that time after AGE [29], PAGE [18,28] and IEF [28,29]. Ashcom and Jacobson [18] detected trypsin and chymotryspin after PAGE with AF-BSA as substrate by a procedure similar to that of Martin et al. [53]. For documentation, high-contrast photographs were made on 35-mm Kodak Technical Pan film No. 2415 through a Kodak No. 4 yellow Wratten gelatine filter. Another group detected cellulases after AGE and IEF [29] and cellulose 1,4,β-cellobiosidases after IEF, PAGE and two-dimensional electrophoresis (PAGE–IEF) [28]. The separation gel was brought into contact with 2% agar gel (0.5–1.0 mm thick) containing 0.14 *M* acetate buffer (pH 4.5) and 2.5 m*M* MU glycoside. The gels were incubated at room temperature for 5–15 min until clear fluorescent bands became visible under UV radiation (355 nm). Then, modifying the usual procedure, the gels were separated and photographed. For taking photographs, the sensitivity of the detection was increased by exposing the gels to ammonia vapour. The authors took replicas with four substrates (all MU derivatives) from the same separating gel [29]. The fluorogenic substrate can also be combined in the detection gel with any of two chromogenic substrates (Ostazin Brilliant Red H-3B hydroxyethylcellulose or Remazol Brilliant Blue R 4-O-methyl-D-glucurono-D-xylan) so that two enzyme activities can be detected simultaneously [28].

3.3.2. Filter-paper overlay technique

Filter-paper overlays have mainly been used after SGE [4,17,57–61], but in some instances also after CAE [62] and after AGE [56,63], to detect esterases and glycosidases. After SGE, enzyme bands have been revealed as follows. Either Whatman filter–paper (No. 1, 3 or 17) was soaked in freshly prepared substrate solution and smoothed firmly on to the cut surface of the gel [4,17,58–60] or the cut surface of the gel was covered with filter–paper and flooded with the appropriate substrate solution [57,61]. After incubation at 37°C or room temperature for 5–15 min (sometimes wrapped in Saran foil), the filter–papers were removed and the gels were viewed under UV radiation. In a study on α-L-fucosidase, Turner et al. [61] incubated for 30–60 min and, after removing the paper, flooded the gel with 85 m*M* glycine carbonate buffer (pH 10.0). For the detection of esterases with 2-naphthyl acetate (2-naphthol revealed by staining) and N-methoxyindoxyl acetate, Seghatchian et al. [58] reported that also for very low-activity components no advantage is gained by leaving the filter-paper in contact for more than 20 min because of the resultant increase in diffusion and background fluorescence. The resolution of esterase D isoenzyme detection with MU esters in acid and neutral pH gels was good for several minutes, necessary for photographing and marking the bands [59]. When staining alkaline gels such as borate gels (pH 8.0–8.6), however, the period of good resolution was found to be very short, owing to the spontaneous hydrolysis of the substrate in contact with the alkaline gel surface. This was minimized by using a stronger acetate buffer (pH 5.2) for the substrate strip (0.5 instead of 0.1 *M*).

After AGE, filter-papers moistened with substrate solution have also been laid on to the gels, followed by incubation as described above. Mar-

tin [56] pointed out that it is very important to observe pH and buffer concentration conditions in studies on acid phosphatase isoenzymes, otherwise the correct quantitative ratio of the isoenzymes is not determined correctly, owing to differences in pH optimum. Ng et al. [62] modified the procedure by incubating the CAE strips between two filter-papers moistened with substrate in buffer. Photographic conditions for fluorescent 4-methylumbelliferone bands have been given by Kühnl et al. [63]: Polaroid MP3 camera, orange filter, black-and-white film type 107, aperture 4.5 at 2 min exposure time.

3.3.3. Cellulose membrane overlay technique

This technique differs from the two aforementioned overlay techniques in the staining taking place always in the overlay membrane, and seldom additionally in the separation gel. The procedure consists of two steps: the preparation of the substrate membranes and the incubation of the gels with the membranes applied on the gel surface followed by observation of the fluorescence on the membranes under UV radiation. This technique seems to be the method of choice within the last 12 years. It has been used mainly after IEF and seldom after CAE [64] and PAGE [39]. Peptidases have very frequently been detected in this way [18,20,30,34,39,65–67], but also phosphatases [23,64,68] and glycosidases [23,44].

The substrate membranes have mainly been prepared as described in detail by Smith [20], who also introduced the designation "enzyme overlay membrane" (EOM) for those membranes. Some of the membranes are commercially available. Cellulose acetate predominates as the membrane material, but cellulose hydrate, cellulose diacetate [68], cellulose nitrate [18,64] and nylon [44] have also been used. The cellulose sheet is washed in distilled water and dried with a cool stream of air. Substrate medium is poured in a prewarmed (45°C) plastic container and the membrane is placed on the surface of the medium. By electrostatic charge the medium is evenly absorbed into the membrane. The membrane is picked up out of the solution and replaced upside down on the surface to absorb the medium. The total immersion time is about 2 min. The membrane is again dried with cool air. The stability of most substrate membranes (depending on the substrate impregnated) was found to be 4–6 months. The substrate membrane should be handled only with forceps. The fluorogenic substrates to be impregnated, at 2 m*M* concentration, are prepared just as they would be for use in a liquid-phase system. They are buffered to the optimum pH of the enzyme with 0.1 *M* buffers. It is also possible to incorporate activating and inhibiting agents of low molecular mass. The preparation of an AF–BSA cellulose nitrate membrane is different [18].

For application, the dry substrate-impregnated membrane is cut to appropriate dimensions, dipped into distilled water for moistening and carefully laid on the surface of the gel. The gel, with the membrane firmly attached, is incubated in a humidified box at 20 or 37°C. The time (in most instances 5–15 min and not exceeding 45 min) and temperature depend on the enzyme, the sample concentration applied to the gel and the substrate impregnated in the membrane. The reaction is observed under a long-wavelength UV lamp. When the optimum fluorescence intensity of the isoenzyme bands is reached, the membrane is removed from the gel and air dried. The contact side of the membrane is photographed within 12 h. The negatives of the photographs of the fluorescent bands also permit an analysis with a visible-light densitometer. Sinha and Gossrau [23] treated the cellulose acetate membranes after incubation on gel with an alkaline solution (e.g., glycine–NaOH buffer or hydrogen carbonate buffer) to obtain maximum fluorescence, when 1-naphthol, 2-naphthol or MU are the fluorogenic leaving groups in substrates for alkaline phosphatase. Stored in a protective plastic cover, the fluorescent products in the membrane are stable for several months. After removal of the impregnated membranes, the separation gels can be stained for proteins with silver stains.

Another cellulose membrane overlay procedure has very recently been reported by Hampton and Rutan [64]. New items are the introduction of a charge-coupled device imaging

system as an improvement over video cameras and densitometers and the kinetic analysis of product formation. Using a fluorogenic substrate, Hampton and Rutan demonstrated the procedure with the example of alkaline phosphatase isoenzymes separated by CAE. After electrophoresis, the electrophoresis membrane was placed on a glass slide within the imaging chamber. The overlay strip, a cellulose nitrate membrane soaked with the substrate, MU phosphate, was placed on the electrophoresis membrane for 15 s and then carefully lifted from the membrane. Consecutive images were taken of the electrophoresis membrane (unlike other cellulose membrane overlay procedures) with a charge-coupled device camera during the time period of the reaction, and kinetic analysis of the data was performed. The method should also be useful for the separation and quantification of other isoenzyme systems. Any system that can produce either an absorbance or a fluorescent signal can be monitored with this method. The relatively short analysis time required should be beneficial for clinical diagnosis.

3.4. Quantification

Quantification of enzyme activity with fluorogenic substrates after elution follows common protocols and will not be discussed here. Information on quantification of hydrolase activity directly on the supporting material is limited, although this technique is commonly used in lactate dehydrogenase and creatine kinase isoenzyme determination [3].

Friedland et al. [41] reported a simple and rapid procedure for screening purposes to detect Tay-Sachs disease carriers in 1970. The disease can be demonstrated by an altered ratio of β-N-acetylhexosaminidase A and B isoenzymes in leukocytes. They electrophoresed the samples by rod gel PAGE and detected the isoenzymes with MU N-acetyl-β-D-glucosamide in the rods. Readings should be made on the same day. The gels were kept moist at 4°C until scanned. For the measurement each gel was placed in a small rectangular quartz cell containing sufficient buffer to prevent air bubbles. The cell was mounted on the door of the Turner automatic scanner unit and the scan of the fluorescent bands was recorded. Measurement of the area under each curve was performed by triangulation, and the results were expressed as percentage of the total area.

Further, a simple and sensitive procedure to scan and quantify zymograms of serum alkaline phosphatases was described by Demetriou and Beattie in 1971 [48]. After incubation of the electrophoresis gel with naphthol AS-MX phosphate, the agarose film was dried at 80°C for 15 min, so that the fluorescent product was stable to light exposure. Under long-wavelength UV radiation, each sample lane was cut for fluorescent scanning. The scans were made with an automatic strip scanner attached to the recording fluorimeter. The light source was a general-purpose UV lamp with a 360-nm primary and 505-nm secondary filter. Aperture settings of 1–30× were adjusted according to the intensity of the fluorescent zones of the electropherograms. Good electrophoretic pattern recordings were obtained with 1 μl of serum and a fluorimeter setting of 10× for sera with alkaline phosphatase levels of 7–30 units. The interpretation was done by planimetric measurements of the zymogram recordings.

Bergerman and Blethen [55] also developed a method for the determination of alkaline phosphatase isoenzymes in 1972. They stated that in fact any enzyme which has a product that fluoresces or can be made fluorescent could be subjected to analysis by this technique. After electrophoresis of serum samples on cellulose acetate strips, the isoenzymes are stained by the agarose gel overlay technique with naphthol AS-MX phosphate as described in Section 3.3. After drying the cellulose acetate strips for about 30 min at room temperature, the fluorescent spots are relatively stable and can be scanned at any time up to 24 h. The strips are cut in half lengthwise to fit the automated reader-door of the Turner Model III fluorimeter. These strips, after being recorded, are stained with Ponceau S and rescanned for their protein distribution. The precision and reproducibility of the method were

reported to be comparable to routine lactate dehydrogenase isoenzyme methods and can be performed in less than 3 h. The suitability for screening large numbers of specimens was discussed.

Naphthol AS-MX liberated from the respective phosphate by alkaline phosphatase after CAE was also scanned by Murayama and Kanno [69]. The calibration graph was shown to be linear in the range 5–640 units/l.

To overcome difficulties with fluorophore stability, Sullivan and Johnson [19] quantified enzymatic activity by photographing the fluorescent bands and scanning the negatives with a laser densitometer. Neprilysin (enkephalinase) isoforms from human kidney or from bovine nuchal ligament were separated by SDS-PAGE and incubated with Dans–D-Ala–Gly–Phe(NO_2)–Gly after removal of SDS. The fluorescent gels were photographed (MP-4 Polaroid camera with a 65A Wratten gelatine filter, illumination at 304 nm), and the negatives were scanned using a Zenith laser densitometer. Noise was reduced by combining traces from multiple scans, and background fluorescence was eliminated by subtraction of phosphoramidone-inhibited control lanes. The areas under the generated curves were related to the amount of enzyme applied to the gels (correlation coefficient not given).

As described in detail in the previous section, Hampton and Rutan [64] recently dealt with the determination of alkaline phosphatase isoenzymes by taking advantage of the combination of electrophoresis followed by indirect staining with a fluorogenic substrate, charge-coupled device imaging system and kinetic analysis. The development of fluorescent bands was followed for a certain time with the charge-coupled device camera. Computerized kinetic analysis was shown to resolve and quantify also overlapped bands. They developed this method because none of other methods used to study alkaline phosphatases is completely satisfactory for the determination of all isoenzymes. Investigations of human serum containing alkaline phosphatase isoenzymes with this method are in progress.

4. Hydrolases detected after electrophoresis

The examples compiled in Tables 2–4 were selected considering differences in the enzyme detected, in the substrate, in the electrophoretic technique used and in the detection procedure applied. The examples show that highly sensitive detection with fluorogenic substrates can be applied after separating the enzymes by any of the usual electrophoresis techniques. Whereas SGE and CAE predominated in the beginning, PAGE, IEF and SDS-PAGE with their better resolution prevailed later. Although AGE was not used to the same extent as were the other techniques, it was used during the whole period. Paper electrophoresis is of minor importance in this field. The enzymes studied first with fluorogenic substrates following electrophoresis mainly belong to the subclasses esterases and glycosidases.

4.1. Esterases

Examples are presented in Table 2. Carboxylesterases, acetylesterases, acetylcholinesterases and cholinesterases have mainly been separated by SGE followed by applying the fluorogenic substrates directly or by filter-paper overlay. Paper electrophoresis [35], PAGE [58] and, later, IEF [23] have also been used. Extraction followed by enzyme assay in solution and the cellulose membrane overlay technique have never been used after SGE. Fluorogenic substrates used for this group of esterases were N-methylindoxyl, N-methoxyindoxyl and 1-naphthyl acetate, naphthol AS derivatives, fluorescein short-chain esters and, above all, MU short- and long-chain esters.

Other esterases such as alkaline and acid phosphatases were also separated by SGE in the beginning, but they and the sulfatases have also been separated by CAE. The enzymes have been visualized after CAE mainly by direct staining and agarose gel overlay. A recent paper deals with the application of cellulose membrane overlay after CAE [64] which is commonly used only after IEF. Substrates used were naphthyl phos-

Table 2
Examples of fluorogenic substrates used for esterase detection

Enzyme	Substrate[a]	Technique[b]	Ref.
Carboxylic ester hydrolases			
Carboxylesterase	MU acetate	SGE/DS	[70]
(EC 3.1.1.1)	Naphthol AS acetate	IEF/CMO	[23]
	Naphthol AS-D acetate	IEF/CMO	[23]
	Naphthol AS-TR acetate	IEF/CMO	[23]
	MU acetate	IEF/CMO	[23]
	MU acetate	SGE/FPO	[17]
	MU propionate	SGE/FPO	[17]
	MU butyrate	SGE/FPO	[17]
	MU heptanoate	SGE/DS	[17]
	MU nonanoate	SGE/DS	[17]
	Fluorescein diacetate	SGE/DS	[17]
	Fluorescein propionate	SGE/DS	[17]
	Fluorescein butyrate	SGE/DS	[17]
	N-Methylindoxyl acetate	SGE/DS	[17]
	MU acetate	AGE/FPO	[63]
	MU acetate	SGE/FPO	[59]
	MU butyrate	SGE/FPO	[59]
	MU heptanoate	SGE/FPO	[59]
	1-Naphthyl acetate	SGE/DS	[59]
	MU caprylate	PE/EAS	[35]
Acetylesterase	MU acetate	SGE/FPO	[17]
(EC 3.1.1.6)	MU propionate	SGE/FPO	[17]
	MU butyrate	SGE/FPO	[17]
	MU heptanoate	SGE/DS	[17]
	MU nonanoate	SGE/DS	[17]
	Fluorescein diacetate	SGE/DS	[17]
Acetylcholinesterase	N-Methoxyindoxyl acetate	SGE/FPO	[58]
(EC 3.1.1.7)	N-Methoxyindoxyl acetate	PAGE/DS	[58]
Cholinesterase	MU butyrate	SGE/FPO	[17]
(EC 3.1.1.8)	N-Methylindoxyl acetate	SGE/DS	[17]
Phosphoric monoester hydrolases			
Alkaline phosphatase	MU phosphate	CAE/CMO	[64]
(EC 3.1.3.1)	MU phosphate	AGE/?	[71]
	Naphthol AS-TR phosphate	IEF/CMO	[23]
	MU phosphate	IEF/CMO	[23]
	Naphthol AS-MX phosphate[c]	CAE/DS	[50]
	Naphthol AS-MX phosphate	CAE/AGO	[55]
	Naphthol AS-MX phosphate	CAE/AGO	[54]
	Naphthol AS-MX phosphate[c]	AGE/DS	[48]
	Naphthol AS-MX phosphate	SGE/DS	[22]
	1-Naphthyl phosphate	SGE/FPO	[4]
	2-Naphthyl phosphate	SGE/FPO	[4]
Acid phosphatase	TFMU phosphate[d]	IEF/CMO	[68]
(EC 3.1.3.2)	MU phosphate	IEF/CMO	[68]
	Naphthol AS-TR phosphate	IEF/CMO	[23]
	MU acetate	AGE/FPO	[56]
	MU acetate	CAE/AGO	[56]
	MU phosphate	IEF/CMO	[23]
	MU phosphate	CAE/AGO	[53]

(*Continued on p. 194*)

Table 2 (*continued*)

Enzyme	Substrate[a]	Technique[b]	Ref.
	MU phosphate	SGE/AGO	[53]
	1-Naphthyl phosphate	SGE/FPO	[60]
	MU phosphate	SGE/FPO	[60]
	MU phosphate	SGE/DS	[40]
Sulfuric ester hydrolase			
Arylsulfatase	MU sulfate	PAGE/DS	[42]
(EC 3.1.6.1)	MU sulfate	CAE/DS	[52]

[a] MU = 4-methylumbelliferyl; TFMU = 4-trifluoromethylumbelliferyl.

[b] AGE = agarose gel electrophoresis; AGO = agarose gel overlay; CAE = cellulose acetate electrophoresis; CMO = cellulose membrane overlay; DS = direct staining; EAS = elution followed by assay in solution; FPO = filter-paper overlay; IEF = isoelectric focusing; PAGE = polyacrylamide gel electrophoresis; PE = paper electrophoresis; SGE = starch gel electrophoresis; ? = not given.

[c] According to the information of the producer (Sigma), α-naphthol AS-MX phosphate, as designated by the authors, has never been produced.

[d] Designated 4-trifluoromethylcoumarin phosphate by the authors.

phates [4,60] and, more frequently, naphthol AS-MX, naphthol AS-TR or MU phosphate for the phosphatases and MU sulfate for the sulfatase.

4.2. Glycosidases

The number of glycosidases detected with fluorogenic substrates (Table 3) is larger than that of the esterases. Here, too, separations by SGE have been used in earlier studies by two groups [57,61,77–80]. Visualization has been achieved by the filter-paper overlay technique. As early as 1970, Friedland et al. [41] separated β-N-acetylhexosaminidases by PAGE and detected the enzymes by a special direct staining procedure (see Section 3.2). In subsequent years, CAE was used to separate glycosidases, followed by filter-paper overlay [62], elution and assay in solution [37] or direct staining [51]. Since the early 1980s, PAGE and, with some delay, IEF have mainly been used for separation, in addition to AGE to a small extent [29,49]. Further, Biely and Markovic [28] described a two-dimensional separation with PAGE in the first and IEF in the second direction. Direct staining has been applied in most instances, but agarose gel overlays and cellulose membrane overlays have also been used. The first application of SDS-PAGE prior to detection of enzymatic activity with fluorogenic substrates has been described for a glycosidase, β-galactosidase [45]. After washing out the SDS, the gel was agitated in MU β-D-galactoside solution. With the exception of naphthol AS-BI β-D-glucuronide and β-D-glucosaminide [23], the substrates used to detect glycosidases always were MU glycosides.

4.3. Peptidases

The fluorimetric detection of peptidases after electrophoresis (Table 4) was introduced much later than that of esterases and glycosidases. In the 1980s, PAGE, IEF and SDS-PAGE had displaced SGE and CAE owing to their considerably better resolution. Hence it is not surprising that the separation by SGE, followed by application of the substrate as an agarose gel sandwich, has been used only once [32]. After PAGE, visualization has mainly been performed by direct application of substrate solutions, which has exclusively been used after SDS-PAGE, but also by extraction followed by enzyme assay in solution and by agarose gel overlay. After IEF, the cellulose membrane overlay technique clearly predominates, but direct staining and extraction prior to enzyme assay have also been used. Fluorogenic substrates employed to detect peptidases are NA, MNA, MCA or

Table 3
Examples of fluorogenic substrates used for glycosidase detection

Enzyme	Substrate[a]	Technique[b]	Ref.
Cellulase (EC 3.2.1.4)	MU β-lactoside	AGE/AGO	[29]
	MU β-lactoside	IEF/AGO	[29]
	MU β-cellobioside	IEF/AGO	[29]
Chitinase (EC 3.2.1.14)	MU β-chitotrioside	PAGE/DS	[72]
	MU β-chitotrioside	PAGE/DS	[73]
	MU β-chitotrioside	IEF/DS	[73]
exo-α-Sialidase (EC 3.2.1.18)	2′-MU-α-D-N-acetylneuraminic acid	PAGE/DS	[74]
	2′-MU-α-D-N-acetylneuraminic acid	PAGE/DS	[44]
	2′-MU-α-D-N-acetylneuraminic acid	IEF/DS	[44]
	2′-MU-α-D-N-acetylneuraminic acid	IEF/NMO	[44]
α-Glucosidase (EC 3.2.1.20)	MU α-D-glucoside	IEF/CMO	[23]
	MU α-D-glucoside	PAGE/DS	[43]
	MU α-D-glucoside	CAE/EAS	[37]
β-Glucosidase (EC 3.2.1.21)	MU β-D-glucoside	IEF/AGO	[28]
	MU β-D-glucoside	PAGE/AGO	[28]
	MU β-D-glucoside	CAE/DS	[51]
	MU β-D-glucoside	SGE/FPO	[57]
α-Galactosidase (EC 3.2.1.22)	MU α-D-galactoside	IEF/CMO	[23]
β-Galactosidase (EC 3.2.1.23)	MU β-D-galactoside	SDS-PAGE/DS	[45]
	MU β-D-galactoside	PAGE/DS	[75]
	MU β-D-galactoside	IEF/CMO	[23]
	MU β-D-galactoside	AGE/DS	[49]
	MU β-D-galactoside	SGE/FPO	[57]
β-Glucuronidase (EC 3.2.1.31)	MU β-D-glucuronide	IEF/CMO	[23]
	Naphthol AS-BI β-D-glucuronide	IEF/CMO	[23]
	MU β-D-glucuronide	SGE/FPO	[57]
α-L-Fucosidase (EC 3.2.1.51)	MU α-L-fucoside	CAE/FPO	[62]
	MU α-L-fucoside	SGE/FPO	[61]
β-N-Acetylhexosaminidase (EC 3.2.1.52)	MU N-acetyl-β-D-glucosaminide	PAGE/DS	[72]
	MU β-chitobioside	PAGE/DS	[72]
	MU N-acetyl-β-D-glucosaminide	PAGE/DS	[73]
	MU β-chitobioside	PAGE/DS	[73]
	MU β-chitotrioside	PAGE/DS	[73]
	MU N-acetyl-β-D-glucosaminide	IEF/CMO	[23]
	Naphthol AS-BI β-D-glucosaminide	IEF/CMO	[23]
	MU N-acetyl-β-D-glucosaminide	PAGE/DS	[41]
	MU N-acetyl-β-D-glucosaminide	SGE/FPO	[57]
Cellulose 1,4-β-cellobiosidase (EC 3.2.1.91)	MU β-cellobioside	IEF/DS	[76]
	MU β-cellobioside	PAGE/DS	[76]
	MU β-lactoside	IEF/AGO	[28]
	MU β-lactoside	PAGE/AGO	[28]

[a] MU = 4-methylumbelliferyl.
[b] NMO = nylon membrane overlay; SDS = sodium dodecyl sulfate; for others see Table 2.

Table 4
Examples of fluorogenic substrates used for peptidase detection

Enzyme	Substrate[a]	Technique[b]	Ref.
Exopeptidases			
Leucyl aminopeptidase (EC 3.4.11.1)	Leu–NA	SGE/AGO	[32]
	Met–NA	SGE/AGO	[32]
	Arg–NA	SGE/AGO	[32]
	Ala–NA	SGE/AGO	[32]
	Phe–NA	SGE/AGO	[32]
Membrane alanyl aminopeptidase (EC 3.4.11.2)	Ala–MNA	IEF/CMO	[27]
	Leu–NA	SGE/AGO	[32]
	Met–NA	SGE/AGO	[32]
	Arg–NA	SGE/AGO	[32]
	Ala–NA	SGE/AGO	[32]
	Phe–NA	SGE/AGO	[32]
Glutamyl aminopeptidase (EC 3.4.11.7)	α-Glu–MNA	IEF/CMO	[27]
Dipeptidyl-peptidase II (EC 3.4.14.2)	Lys–Ala–TFMCA	IEF/CMO	[65]
Dipeptidyl-peptidase III (EC 3.4.14.4)	Arg–Arg–NA	SGE/AGO	[32]
	Leu–Ala–NA	SGE/AGO	[32]
Dipeptidyl-peptidase IV (EC 3.4.14.5)	Ala–Pro–TFMCA	IEF/CMO	[66]
	Gly–Pro–MNA	IEF/CMO	[27]
	Gly–Pro–MCA	IEF/CMO	[27]
	Gly–Pro–TFMCA	IEF/CMO	[27]
	Gly–Pro–MNA + nitrosalicylaldehyde	IEF/DS	[27]
Endopeptidases			
Chymotrypsin (EC 3.4.21.1) and chymotrypsin-like	AF–BSA	PAGE/AGO	[18]
	Ser–Tyr–TFMCA	PAGE/CMO	[39]
	Ser–Tyr–TFMCA	PAGE/EAS	[39]
	Suc–Leu–Leu–Val–Tyr–MCA	PAGE/DS	[81]
Trypsin (EC 3.4.21.4) and trypsin-like	AF–BSA	PAGE/AGO	[18]
	Boc–Glu–Lys–Lys–MCA	SDS-PAGE/DS	[46]
	Z–Gly–Gly–Arg–TFMCA	IEF/CMO	[30]
	Z–Arg–TFMCA	IEF/CMO	[30]
	Boc–Val–Pro–Arg–MCA	PAGE/DS	[82]
Tissue kallikrein (EC 3.4.21.35)	D-Val–Leu–Arg–TFMCA	IEF/CMO	[67]
	D-Val–Leu–Arg–TFMCA (+ SBTI)	IEF/CMO	[34]
	D-Val–Leu–Arg–MCA	IEF/CMO	[20]
	D-Val–Leu–Arg–MNA	IEF/DS	[27]
	D-Val–Leu–Arg–MNA + nitrosalicylaldehyde	IEF/DS	[27]
Serine endopeptidases	D-Val–Leu–Arg–TFMCA	IEF/CMO	[30]
Cathepsin B (EC 3.4.22.1)	Bz–Val–Lys–Lys–Arg–TFMCA (+aprotinin)	IEF/CMO	[34]
	Z–Ala–Arg–Arg–MNA	IEF/CMO	[27]
	Z–Phe–Arg–MCA	IEF/DS	[83]
	Z–Arg–Arg–MCA	IEF/DS	[83]
Cathepsin L (EC 3.4.22.15)	Z–Phe–Arg–MCA	IEF/DS	[83]
Cysteine endopeptidases	Z–Arg–Arg–MCA	SDS-PAGE/DS	[31]
	Z–Phe–Arg–MCA	SDS-PAGE/DS	[31]
	Boc–Val–Leu–Lys–MCA	SDS-PAGE/DS	[31]
	Suc–Ala–Phe–Lys–MCA	SDS-PAGE/DS	[31]
	Pro–Phe–Arg–MCA	SDS-PAGE/DS	[31]
	Leu–Val–Tyr–MCA	SDS-PAGE/DS	[31]
	Bz–Phe–Val–Arg–MCA	SDS-PAGE/DS	[31]
	Suc–Leu–Tyr–MCA	SDS-PAGE/DS	[31]
Tc 120 proteinase (alkaline cysteine proteinase)	Z–Gly–Gly–Arg–MCA	SDS-PAGE/DS	[47]
	Z–Arg–MCA	SDS-PAGE/DS	[47]
	Z–Phe–Arg–MCA	SDS-PAGE/DS	[47]
	Glt–Gly–Arg–MCA	SDS-PAGE/DS	[47]

Table 4 (*continued*)

Enzyme	Substrate[a]	Technique[b]	Ref.
Cathepsin D (EC 3.4.23.5)	AF-BSA	IEF/CMO	[18]
Neprilysin (EC 3.4.24.11)	Dans-D-Ala-Gly-Phe(NO_2)-Gly	SDS-PAGE/DS	[19]
Multicatalytic endopeptidase complex (EC 3.4.99.46)	Z-Val-Leu-Arg-MNA	PAGE/EAS	[38]
Alkaline proteinase	Bz-Arg-MCA	PAGE/DS	[84]
High-molecular-mass proteinase II/III	Bz-Val-Gly-Arg-MCA	PAGE/EAS	[36]
	Bz-Val-Gly-Arg-MCA	IEF/EAS	[36]
	Suc-Ala-Ala-Phe-MCA	PAGE/EAS	[36]
	Suc-Ala-Ala-Phe-MCA	IEF/EAS	[36]

[a] AF-BSA = acetamidofluorescein-bovine serum albumin; Boc = *tert*-butyloxycarbonyl; Bz = N^2-benzoyl; Dans = 5-dimethylaminonaphthalene-1-sulfonyl (dansyl); Glt = glutaryl; MCA = 4-methylcoumarinyl-7-amide (also abbreviated to AMC: 7-amino-4-methylcoumarin); MNA = 4-methoxy-2-naphthylamide; NA = 2-naphthylamide; Phe(NO_2) = *p*-nitrophenylalanyl; SBTI = soybean trypsin inhibitor (Kunitz); Suc = succinyl; TFMCA = 4-trifluoromethylcoumarinyl-7-amide (also abbreviated to AFC: 7-amino-4-trifluoromethylcoumarin); Z = benzyloxycarbonyl (also used: Cbz = carbobenzoxy).

[b] See Tables 2 and 3.

TFMCA derivatives. Chymotrypsin, trypsin and cathepsin D have also been detected with AF-BSA, a broad spectrum self-quenched substrate (see Section 2.1), and neprilysin has been revealed with Dans-D-Ala-Gly-Phe(NO_2)-Gly, an intramolecularly quenched substrate.

5. Application areas

The methods discussed in this review have been used in many areas. Examples of application to clinical, biological and food chemistry are discussed below.

5.1. Clinical chemistry

Already in 1969, Johnson [22] reported on the separation of serum alkaline phosphatase isoenzymes by SGE and on their detection with naphthol AS-MX phosphate. All normal sera exhibited at least one isoenzyme, and few sera contained all six isoenzymes that have been demonstrated. Johnson discussed a possible correlation between the isoenzymes present, their relative activity and the disease state such as severe liver disease or formation of bony metastases. The work was extended by Elevitch et al. [85], who found characteristic mobility patterns of alkaline phosphatases, using AGE and the aforementioned substrate, in extracts from liver, bile, bone, placenta, kidney, large and small intestine mucosa and leukocytes. Sera from 116 patients selected for abnormally high alkaline phosphatase activities were analysed and classified according to the organ extract patterns. Each serum fell into one of eight categories. When compared with clinical, other biochemical, and tissue findings, these categories clearly distinguished 68 patients with various hepatobiliary disorders from 41 patients with several osteoblastic diseases of bone.

Recently, a method combining kinetic analysis with charge-coupled device imaging to determine alkaline phosphatase isoenzymes has been reported [64] (see Section 3.4). Investigations to correlate human serum isoenzyme activities with human diseases are in progress.

Kidney damage induced in rats by injections of various nephrotoxic agents caused a marked increase in the urinary excretion of β-glucosidase and the appearance of a new isoenzyme in urine [77]. The new urinary isoenzyme was detected with MU β-D-glucoside after SGE. It seemed to be identical with its counterpart in the kidney. It was suggested that it arises in the urine as a result of renal tubular breakdown. Similarly, the renal β-N-acetylhexosaminidase isoenzyme, not normally found in the urine, was excreted by patients having damaged kidneys [78], whereas the corresponding renal β-glucosidase isoenzyme was not found in urine from patients who had renal trauma (opposite to the rat). The appearance of the renal β-N-acetylhexosaminidase isoenzyme in the urine was paralleled by an increase in urinary β-N-acetylhexosaminidase ex-

cretion, which was small in patients undergoing minor renal trauma but generally large in human subjects undergoing severe surgical renal trauma. In a subsequent paper [79], similar results were reported for patients with renal disease and, in addition, two cases were found in which the renal β-galactosidase isoenzyme, having β-glucosidase activity, was also present in the urine. The apparent absence of this enzyme in urines from other patients may be explained by the need to store samples before analysis. The findings do not indicate, however, that abnormal enzyme excretion is in any way diagnostic of a particular form of renal disease. The abnormal excretion was thought to be a function more of cell damage in general than of a particular disease process within the kidney.

Tay-Sachs disease carriers have been identified by a lower ratio of leukocyte β-N-acetylhexosaminidase isoenzymes A to B (mean 49% A: 51% B) than found in normal individuals (66% A : 34% B) [41]. These ratios have been determined by PAGE, direct staining with MU N-acetyl-β-D-glucosaminide and fluorimetric scanning of the gels. The absence of the isoenzyme A in leukocytes of Tay-Sachs disease patients has also been confirmed by this method.

The isoenzymes of human α-L-fucosidase have been separated by SGE and detected by indirect staining with MU α-L-fucoside [61]. None of the isoenzymes was detectable in lymphoid cell lines, serum or cultured fibroblasts from a patient with fucosidosis, an inborn error of metabolism. Fucosidosis carriers have been identified by determinating the ratio of arylsulfatase to α-L-fucosidase activity in urine [62]. The ratios for fucosidosis carriers were higher than those for normal individuals. Although the determinations have been done with the appropriate fluorogenic substrates in solution, a technique similar to that described to identify Tay-Sachs disease carriers should be possible.

Arylsulfatase isoenzymes have been detected in extracts of leukocytes, cultured fibroblasts and amniotic fluid cells after CAE with MU sulfate [52]. In preparations of fibroblasts from patients with late infantile and adult-onset types of metachromatic leukodystrophy, one isoenzyme band was absent. The applicability of the method to the prenatal diagnosis of the disease has been discussed.

5.2. Biological chemistry

The detection of hydrolase activity with fluorogenic substrates after electrophoretic separation has also been used to control the efficiency of genetic engineering. The *lacZ* gene of *Escherichia coli*, coding for β-galactosidase, has been used as reporter gene in these studies. The demonstration of the formation of a hybrid β-galactosidase by electrophoresis and specific staining for β-galactosidase activity was used to ascertain the expression of chimeric genes in mammalian cells [49] and plant cells [45,75].

In another study, PAGE and IEF followed by direct staining were used to characterize the cellulose 1,4-β-cellobiosidase of *Clostridium thermocellum* produced by a recombinant *E. coli* strain [76].

Polymorphic forms of human erythrocyte acid phosphatase previously thought to be peculiar to the red cell have been identified in a variety of other human tissue extracts, and also in cultured fibroblasts, using SGE and indirect staining with MU phosphate [60]. The existence of erythrocyte acid phosphatase isoenzymes in human cultured cell lines provided a new marker which could be used in human somatic cell genetic studies. Erythrocyte acid phosphatase isoenzyme patterns have also been used for phenotyping bloodstains by SGE and MU phosphate staining with very good results [86]. Bargagna [87] applied the method to typing a population of 495 individuals from Tuscany, Italy, and found it very satisfactory for forensic purposes. Martin [56] improved the method by introducing AGE and discussed the influence of staining conditions.

A new human esterase, designated esterase D, has been discovered using MU acetate and MU butyrate as substrate after SGE [59]. Three different commonly occurring electrophoretic types of esterase D have been identified. Esterase D isoenzymes were detected in all the human tissues examined, including adult post-mortem

tissues, foetal livers, cultured fibroblasts and lymphocytoid cells. Family studies of 179 families with 410 offsprings showed that they are genetically determined by two alleles at an autosomal locus. It was suggested that these isoenzymes are useful new markers in somatic cell hybridization experiments and population studies. Two years later, Coates et al. [17] showed that the thirteen sets of esterase isoenzymes are determined by at least nine different structural gene loci. The isoenzyme patterns of esterase D have been used for typing dried human bloodstains using SGE and MU butyrate [88]. The three electrophoretic patterns of esterase D have been demonstrated also in Amazonian primates (*Cebus apella*) [89].

Fluorogenic peptide substrates have been used to detect the two coagulation factors II and X after AGE [90]. The zymogens have been localized after activation to thrombin and factor Xa, respectively. The differential detection of factors II and X can be achieved by the selective use of either different peptidases substrates, different activating agents or different anticoagulants. For example, factor II can be specifically activated with Australian taipan snake venom and revealed with Boc–Val–Pro–Arg–MCA, or can be collected on heparin, which coagulates factor X, activated with Russel's viper venom, which activates the two zymogens, and localized with Boc–Ile–Glu–Gly–Arg–MCA. Factor X, on the other hand, can be activated specifically with factor X activating enzyme and detected with Boc–Ile–Glu–Gly–Arg–MCA, or can be activated with Russel's viper venom in the presence of hirudin, which inhibits thrombin, and detected with the same substrate. It was pointed out that this approach can be extended to other factors, where their specific peptide cleavage sequence is known.

5.3. Food chemistry

The same group of substrates has recently been used to localize antinutritive food proteins after various electrophoreses [33]. The antinutrients, inhibitors of the digestive endopeptidases trypsin and chymotrypsin, were detected by a negative staining technique that also discriminates between the two inhibitor types due to the specificity of the substrates. After electrophoresis, slab gels were first incubated with the enzyme (bovine trypsin, bovine chymotrypsin or human duodenal juice) at 37°C, and then covered with the respective substrate membrane (Z–Arg–MCA or Z–Gly–Gly–Arg–TFMCA for trypsin, Ala–Ala–Phe–MCA for chymotrypsin, on a cellulose acetate membrane) and incubated at room temperature while being observed under UV radiation. Dark blue inhibitor bands on a light blue fluorescent background were obtained with Z–Arg–MCA/trypsin and Ala–Ala–Phe–MCA/chymotrypsin, whereas Z–Gly–Gly–Arg–TFMCA resulted in dark inhibitor bands on a green fluorescent background. The "inhibitor overlay membrane" (IOM) technique was shown to be applicable to IEF with carrier ampholytes and immobilized pH gradients, pore-gradient PAGE and SDS-PAGE. Using this technique following IEF, more than 30 inhibitor bands with activity against human and bovine trypsin and chymotrypsin have been detected in fenugreek seeds [91] and 23 inhibitors active against the four aforementioned enzymes in lentils [92].

6. Conclusions

The electrophoresis of proteins has now reached a high standard in both performance and separation. This is especially true for IEF and SDS-PAGE. Detection methods based on specific properties of the proteins such as enzymatic activity will surely gain even more attention. Thus, the introduction of sensitive and specific fluorogenic substrates for further hydrolases is to be expected. Among the fluorogenic substrates used today for hydrolases, those with 4-methylumbelliferone and 4-methyl- or 4-trifluoromethyl-7-aminocoumarin as leaving group seem to be the most promising, also with regard to toxicological aspects.

The improvement of quantification also deserves further attention. From our point of view, even the quantification of proteins by staining after electrophoresis is not possible in all in-

stances. The quantification of enzymatic activity after electrophoresis is, without doubt, more complicated. Thus, it is not very probable that correct absolute values will ever be obtained. However, the determination of relative data yielding activity ratios should be possible. A promising way seems to be the combination of an imaging system with computerized kinetic analysis of the data.

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