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Enzyme amplification as detection tool in continuous-flow systems I. Development of an enzyme-amplified biochemical detection system coupled on-line to flow-injection analysis

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Abstract

The on-line coupling of flow-injection analysis (FIA) to an enzyme-amplified biochemical detection (EA-BCD) system is described. The aim of this study is the development of a detection system able to detect biotin-containing compounds at low concentration levels. The detection system is based on the interaction of biotin with enzyme-labeled affinity proteins. Biotin possesses a high affinity to both streptavidin and anti-biotin Fab fragments, which are both tested. Several biotin derivatives are available with different reactive probes, which can be used to label analytes of interest. Therefore, biotin acts as a universal probe for the enzyme-amplified biochemical detection. Alkaline phosphatase (AP) was used as enzyme label. Several parameters, such as substrate type and concentration, concentration of enzyme-labeled affinity protein, reaction time and reaction temperature were examined. Biotin aminocaproic acid was used as a model compound. In addition to biotin aminocaproic hydrazide, other biotinylation reagents were also examined. With fluorescence detection of the enzyme-generated product, a mass detection limit of 1 fmol was achieved.

Keywords: Enzyme amplification; Flow injection analysis; Detection, FIA; Biotin; Alkaline phosphatase

1. Introduction

Over the past few decades there has been an increasing interest in the application of enzymes for detection of analytes at low concentration levels. The major advantage of enzymatic detection systems is the amplification step caused by the conversion of manifold substrate molecules to produce detectable molecules by each enzyme. Monitoring of the enzyme product can be done by UV absorption, fluorescence, luminescence or electrochemical detection [1–3]. Many enzyme-amplified detection systems are based on the interaction of enzyme-labeled antibodies with an antigen. Enzyme immunoassays are comparable with radioimmunoassays with respect to sensitivity but have the advantage of using non-radioactive labels.

The most commonly used assay is the sandwich enzyme-linked immunosorbent assay (ELISA) [4]. This assay requires two antibodies that bind non-

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overlapping moieties of the analyte. The first antibody is immobilized on a solid phase. After incubation of the analyte with the immobilized antibody a second, enzyme-labeled antibody is allowed to bind with the other moiety of the analyte. After removal of excess second antibody, substrate is added which is converted to a detectable product. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the most commonly used enzymes due to their stability and the wide range of substrates available [1]. With the use of substrates, which are converted into fluorescent or luminescent products, limits of detection (LODs) at the amol level have been obtained [5,6].

Enzyme assays are usually performed in microtiterplates for the simultaneous analysis of multiple samples. However, batch assays are laborious and long incubation times are necessary for sensitive detection. The accuracy of batch assays, compared to high-performance liquid chromatography (HPLC) or flow-injection analysis (FIA), is rather poor (inaccuracy~15–20%) [1,7]. Another limitation of batch assays is the impossibility to distinguish between multiple cross-reactive compounds in one sample. An additional separation is needed to separate different analytes of interest. However, coupling of a separation technique to microtiterplates is difficult to automate and will give erroneous results. Therefore, the on-line coupling of enzyme-amplified biochemical detection (EA-BCD) to FIA and HPLC is a challenging task.

Gunaratna and Wilson [8] developed an on-line EA-BCD system coupled to FIA for the analysis of difluoromethylornithine (DFMO). A polyclonal antibody labeled with HRP was incubated off-line with DFMO followed by FIA. Excess affinity protein was separated from the analyte-bound affinity protein by means of an immobilized DFMO column. Next, substrate was added, which was converted by the enzyme label. An LOD of 0.2 fmol was achieved using 3-(*p*-hydroxyphenyl) propionic acid as substrate. Recently a non-competitive flow-injection enzyme immunoassay was introduced for digoxin and digoxigenin [9,10].

Instead of raising antibodies to analytes of interest, as described in the system used by Gunaratna and Wilson, a more general approach consist of the labeling of analytes with a probe, which interacts with enzyme-labeled affinity proteins. Development of an EA-BCD system that responds to a universal probe instead of an analyte will result in a universal detection system able to detect all analytes, which can be labeled with the universal probe. The preparation and labeling of antibodies to the analytes of interest, which is a laborious and expensive procedure, can be avoided with the use of a universal probe.

A suitable universal probe is biotin, which interacts with streptavidin and avidin. Biotin is a small, polar compound, which consist of a bicyclic urea structure with a sulfur atom in a thio–ether linkage [11]. To the biotin moiety, a valeric side chain is attached. Other reactive probes can replace the carboxylic functionality of the valeric side chain. Streptavidin and avidin are proteins, which possess four binding sites with a very high affinity for biotin. The interaction with the biotin moiety with a K_D of $1 \cdot 10^{-15}$ mol/l results in one of the strongest non-covalent binding known [12,13]. However, the valeric side chain is not involved in the interaction with the affinity proteins. Furthermore, biotin derivatives are available with different reactive groups attached to the valeric side chain, which can react to primary or secondary amines, aldehydes, ketones and carboxylic groups [14,15]. Derivatization of an analyte with biotin, via the valeric acid side chain, will not change the biological behavior of biotin and will not significantly affect the interaction of biotin with the affinity proteins [12,13]. Biotinylation procedures are widely used and are described in more detail elsewhere [16].

Several BCD systems coupled to FIA, for the analysis of biotin, were developed during the last decade. Oosterkamp et al. [17] described a homogeneous as well as a heterogeneous BCD system coupled to FIA for the detection of biotin. In the homogeneous set-up, which was previously described by Smith-Palmer et al. [18], fluorescein-labeled streptavidin was incubated on-line with biotin, which leads to an enhancement of the fluorescence. In a heterogeneous set-up, an immobilized biotin column removed excess fluorescein-labeled streptavidin, whereas the biotin-bound fraction passes the trapping column unretained. In this way, an LOD of 200 fmol was achieved. Instead of an affinity column, a hollow fiber module can be

applied to separate free and streptavidin-bound biotin [19]. With the use of a hollow fiber with a molecular mass cut-off of 50 000, free biotin was separated from the biotin-bound fraction due to the high molecular mass of streptavidin (60 000 [12]).

Both BCD systems described above, offer the possibility to analyze compounds which are labeled with biotin. However, the sensitivity of BCD using fluorescein as label is much lower compared to EA-BCD [8–10]. Therefore, the combination of a BCD system, which respond to biotin containing compounds, and enzyme-amplified detection will offer the possibility to detect analytes at low concentration level after pre-column derivatization with biotin.

In the present study, we present an analytical technique, which integrates biotinylation, the separation of cross-reactive compounds and post-column EA-BCD in a single, fully automated hardware set-up. The general procedure involves four important sub-procedures, biotinylation, sample clean-up, separation of cross-reactive compounds and EA-BCD. In a first step, analytes are biotinylated using suitable biotinylation reagents. Subsequently the excess of biotinylation reagent is removed using selective sample pretreatment procedures based on solid-phase extraction (SPE). Biotinylated, cross-reactive analytes are separated by reversed-phase HPLC and detected by an EA-BCD system based on streptavidin as affinity protein. Alternatively, anti-biotin Fab fragments are employed.

The present paper describes the final part of the analytical technique depicted above, i.e., the development of a generally applicable, continuous-flow biochemical detection system based on enzyme-labeled streptavidin or anti-biotin Fab fragments as reporter molecules. Alkaline phosphatase was used as enzyme label. The influence of several parameters, such as buffer composition, substrate, reaction time and reaction temperature were considered. Biotin aminocaproic hydrazide (BACH) was used as a model compound. This compound can be used to label analytes of interest via their aldehyde or ketone group in future studies. In addition to BACH also other biotinylation probes were examined. The integration of the continuous-flow EA-BCD in the overall analytical technique is described in a subsequent paper [16].

2. Experimental

2.1. Chemicals

BACH and biotin were purchased from Sigma (St. Louis, MO, USA). Biotin hydrazide (BH), biotin *N*-succinimidyl ester (NHS-biotin) and biotinyl lysine (B-lys) were acquired from Fluka (Zwijndrecht, The Netherlands). 4-Methylumbelliferyl phosphate (MUP), 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) and fluorescein diphosphate (FDP) were obtained from Molecular Probes (Eugene, OR, USA). Immunopure alkaline phosphatase-labeled streptavidin (S-AP) and sulfo succinimidyl-6-(biotinamido)-hexanoate (S-NHS-biotin) came from Pierce (Rockford, IL, USA). Sodium acetate, magnesium chloride hexahydrate, sodium chloride, sodium nitrate, glycine and Tween 20 were purchased from Merck (Darmstadt, Germany). Tris-(hydroxymethyl)-aminomethane (Tris) and sodium cyanoborohydride were acquired from Aldrich (Steinheim, Germany). Methanol and diethanolamine were from Mallinckrodt Baker (Deventer, The Netherlands). Acetonitrile (HPLC grade) was purchased from Rathburn (Walkerburn, UK). Aldehyde-activated Poros 20, Attophos substrate set, AP and alkaline phosphatase-labeled Fab fragments from monoclonal anti-biotin antibody (Fab-AP) were from Boeringer Mannheim (Mannheim, Germany). All aqueous solutions were prepared with water purified with a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Apparatus

A microgradient pump from Brownlee (Applied Biosystems, Santa Clara, CA, USA) was used for delivery of the mobile phase for FIA. The pump was connected to an Aspec XL autosampler equipped with a 401C dilutor from Gilson (Villiers-Le-Bel, France) and a six-port injection valve (Rheodyne, Cotati, CA, USA). The 401C dilutor was used to load the injection loop (1 μl or 5 μl). A Gilson minipulse 3 peristaltic pump was used for substrate transfer. A 402 dilutor from Gilson was used for delivery of enzyme-labeled affinity proteins in carrier buffer. Detection was performed with a Jasco (Tokyo, Japan) FP920 fluorescence detector. All

Gilson equipment was controlled by Gilson 719 software via a Gilson 506C interface module. Data acquisition was performed by Gilson 715 software via the same interface.

2.3. Immobilization of biotin and preparation of the immobilized biotin column

Biotin was immobilized on Poros according to the following procedure. A 20-mg amount of BACH was dissolved in 3 ml 0.1 mol/l sodium acetate (pH 5.6) and 1 ml acetonitrile. Forty mg aldehyde-activated Poros and 4 mg sodium cyanoborohydride was added. Sodium cyanoborohydride was used to reduce the hydrazone bonding, resulting in a highly stable bonding between BACH and Poros. The reaction mixture was allowed to react for 24 h in the dark at room temperature under continuous stirring. Next the suspension was centrifuged at 1000 *g* for 5 min. The supernatant was removed and 3 ml 0.1 mol/l sodium acetate (pH 5.6) was added. The washing procedure was repeated four times. To quench the residual aldehyde functionality, 2 ml 0.2 mol/l Tris was added. After 2 h of continuously stirring, the suspension was centrifuged at 1000 *g* for 5 min and the supernatant was removed. Finally a 5×2 mm I.D. column was slurry-packed with immobilized biotin and the column was rinsed with 2 ml water and 2 ml methanol. The immobilized biotin as well as the packed column was stored in the refrigerator prior to use.

2.4. Substrate optimization

To establish optimum condition for the enzyme–substrate reaction, a FIA system for the injection of alkaline phosphatase (5 μl injection) was set up. To the FIA carrier (0.2 ml/min, methanol–water, 10:90, v/v), substrate was added via a mixing union at a flow-rate of 0.35 ml/min. The substrate was converted by alkaline phosphatase in a PTFE-knitted reaction coil of 850 μl (500 μm I.D.) at ambient temperature. All products were measured with fluorescence detection. Stock solutions of MUP and DiFMUP were prepared by dissolving 1 mg substrate in 1 ml acetonitrile. A 5-mg amount of FDP was dissolved in 2 ml 0.1 mol/l Tris buffer (pH 7.0) and stored in the freezer prior to use. The Attophos stock solution was prepared according to the following procedure. To 36 mg Attophos, 60 ml of Attophos buffer was added, containing 2.4 mol/l diethanolamine (DEA), 0.057 mmol/l magnesium chloride, 0.005% sodium azide, pH 10.0. The final concentration of the substrate solution was 1 mmol/l. The substrate solution was stored in the freezer prior to use. The substrate buffers are summarized in Table 1. To all substrate buffers, 0.5% (v/w) Tween was added to avoid non-specific binding of AP.

2.5. Enzyme-amplified biochemical detection coupled on-line to FIA

The scheme of the EA-BCD system is given in Fig. 1. BACH was injected via a 1-μl loop. To the FIA effluent (50 μl/min, methanol–water, 10:90, v/v), S-AP or Fab-AP in carrier buffer was added via a mixing union at a flow-rate of 170 μl/min. The carrier buffer consists of 20 mmol/l Tris, 150 mmol/l sodium chloride, 150 mmol/l sodium nitrate and 0.5% (v/w) Tween 20. The pH of the carrier buffer was 7.5. The interaction between the analytes and the affinity proteins was performed in a PTFE-knitted reaction coil with a volume of 351 μl (250 μm I.D.) resulting in a reaction time of 96 s. The excess affinity protein was separated from the bound fraction by means of a 5×2 mm I.D. column, containing immobilized biotin. After the separation of the bound and the unbound fraction, substrate was added via a second mixing union at a flow-rate of 90 μl/min. The substrate buffer consists of 10 μmol/l Attophos in 0.1 mol/l DEA, pH 9.9. The enzyme–substrate reaction was performed in a 955 μl PTFE-knitted reaction coil (500 μm I.D.) at 50°C, followed by fluorescence detection. The affinity protein solutions and substrate solutions were prepared freshly every day.

3. Results and discussion

3.1. Assay design

A scheme of the continuous-flow system coupled to EA-BCD is shown in Fig. 1. BACH, which served as an analyte, was incubated on-line with enzyme-labeled affinity protein in a reaction coil (C1).

Table 1
Characteristics and results of the investigated substrates

Characteristic	Substrate			
	MUP	DiFMUP	FDP	Attophos
Buffer composition	0.1 mol/l Glycine, 1 mmol/l $MgCl_2$ (pH 9.0)	0.1 mol/l Glycine, 1 mmol/l $MgCl_2$ (pH 9.0)	0.1 mol/l Tris, 0.1 mol/l NaCl, 1 mmol/l $MgCl_2$ (pH 8.5)	0.1 mol/l DEA (pH 9.9)
Excitation wavelength	358 nm	358 nm	488 nm	440 nm
Emission wavelength	452 nm	452 nm	520 nm	550 nm
Stokes shift	Large	Large	Low	Large
Native fluorescence	High	High	Low	Low
Stability	Low	Low	Mean	High
Fluorescence quantum yield	0.63	0.89	0.92	Unknown
Optimum concentration	1 μmol/l	1 μmol/l	1 μmol/l	10 μmol/l
LOD alkaline phosphatase ($S/N=3$)	190 amol	38 amol	100 amol	8 amol

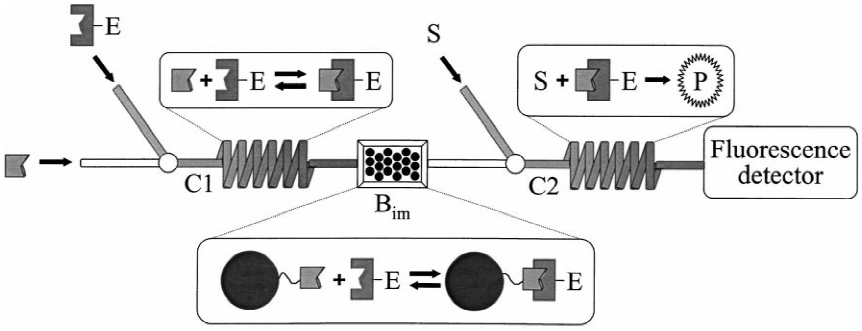

Fig. 1. Schematic representation of the enzyme-amplified biochemical detection. =biotin-containing compounds (FIA effluent, 50 μl/min), E=enzyme-labeled streptavidin (170 μl/min), E=biotin–enzyme-labeled affinity protein complex. C1=reaction coil 1 (351 μl, 250 μm I.D.), B_{im}=immobilized biotin column (5×2 mm I.D.), =immobilized biotin on Poros, S=substrate (90 μl/min), C2=reaction coil 2 (955 μl, 500 μm I.D.), =fluorescent product. For conditions, see text.

Ideally, all BACH will interact with the excess affinity protein before reaching the immobilized biotin column (B_{im}). However, the completeness of the incubation is determined by the excess of affinity protein and incubation time. Next, unbound affinity protein is trapped to the immobilized biotin column (B_{im}). Theoretically, only biotin-bound enzyme-labeled affinity protein passes the immobilized biotin column. Therefore, the binding efficiency of the immobilized biotin column is a crucial part of the system. Finally, substrate is added which is converted to a fluorescent product in the second knitted reaction coil (C2). The use of enzymes as a label has the advantage of production of multiple detectable molecules by each enzyme label, which passes the immobilized biotin. The amplification of the signal depends of the turnover number of the enzyme and the enzyme–substrate reaction time.

3.2. Choice of substrate

An ideal substrate for enzymatic fluorescence detection in flow assays yields a highly fluorescent, soluble product. Furthermore, the optical properties of the substrate should be significantly different from the product. Ideally, the substrate concentration should be high enough for the enzymes to work at the maximum reaction velocity, which results in a maximum amplification. Unfortunately, a higher substrate concentration will also result in a higher background [9] and the optimum concentration has to be determined for each substrate. Four different substrates were examined by FIA of alkaline phosphatase. To the FIA carrier, substrate in an appropriate buffer (see Table 1) was added, which was converted by the injected amount of alkaline phosphatase in a knitted reaction coil. The enzyme–substrate reaction time was 1.5 min. The substrate concentration and characteristics of the fluorescent products are summarized in Table 1.

MUP and DiFMUP are widely applied substrates that are dephosphorylated by alkaline phosphatase to 4-methylumbelliferone (MU) and 6,8-difluoro-4-methylumbelliferone (DiFMU), respectively. Both products have the same excitation and emission characteristics (see Table 1).

However, the fluorescence quantum yield of DiFMU is higher than that of MU (0.89 versus 0.63, respectively) [15]. A disadvantage of both substrates is their low stability. Both substrates are slowly dephosphorylated even in absence of enzyme resulting in a continuously increasing baseline. A second limitation is the relatively high background caused by native fluorescence of the substrates. An optimum substrate concentration of 1 μmol/l for both substrates was found. Higher substrate concentration lead to a background signal, which can not be compensated by the detector used. With the use of MUP as substrate an LOD of 190 amol alkaline phosphatase was determined ($S/N=3$). When using DiFMUP, an LOD of 38 amol alkaline phosphatase was achieved ($S/N=3$). The lower LOD for DiFMUP is the result of a higher fluorescence quantum yield of the fluorescent product.

A very sensitive substrate for alkaline phosphatase is FDP, which is non-fluorescent. The resulting product, fluorescein, has a fluorescence quantum yield of 0.92 at high pH [15]. The high optimum pH of alkaline phosphatase also enhances the fluorescence of fluorescein. The substrate solution was stable for at least one day when protected from light. A limitation of fluorescein is the small Stokes shift (32 nm) resulting in a relatively high noise with the fluorescence detector used. The main problem is the use of Tween 20, which was added to avoid non-specific binding of the enzyme. Tween 20 quenched the fluorescence at the wavelengths used to detect fluorescein resulting in a lower signal-to-noise ratio (S/N). An LOD of 100 amol alkaline phosphatase was achieved ($S/N=3$), using a substrate concentration of 1 μmol/l. A higher substrate concentration increases the signal. However, the background and noise was also increased with a higher substrate concentration, resulting in a lower (S/N).

The alkaline phosphatase activity can also be monitored when Attophos is applied as a substrate. Attophos was introduced by Cano et al. and is converted to the fluorescent Attofluor by alkaline phosphatase [20]. An advantage of Attofluor is the relative large Stokes shift of 110 nm. Furthermore, Attophos fluoresces very weakly at the wavelengths used for the detection of Attofluor (see Table 1). The Attophos solution was stable when stored in the dark. Because of the low background, a higher substrate concentration can be applied compared to the other investigated substrates.

With a higher substrate concentration, the production of Attofluor by the enzyme will be higher, resulting in a more sensitive detection of alkaline phosphatase. The LOD was 8 amol ($S/N=3$) when using 10 μM substrate which was the lowest LOD achieved with the investigated substrate. Therefore, Attophos was used as substrate during further experiments.

3.3. Efficiency of the immobilized biotin column

A crucial part of the EA-BCD system is the separation of free and biotin-bound enzyme-labeled affinity protein prior to fluorescence detection by means of immobilized biotin. The purity of the enzyme-labeled affinity proteins and the capacity of the immobilized biotin column determine the binding efficiency of the immobilized biotin. Impure material may contain free enzymes or enzyme-labeled affinity proteins, which has decreased affinity for biotin, for example due to steric hindrance. These impurities will pass the immobilized biotin column unretained and will increase the background dramatically.

The binding efficiency of the immobilized biotin column was investigated with the EA-BCD system by first omitting the immobilized biotin column (Fig. 1). Carrier and substrate buffer were both pumped. After 10 min, Attophos was added to the substrate buffer to give a final concentration of 10 μmol/l, which resulted in a slight increase of the background (see Fig. 2) induced by the substrate. After 20 min, Fab-AP was added to the carrier buffer to give a final concentration of 7.5 units/liter (U/l). The conversion of the substrate by Fab-AP leads to a strong increase of the fluorescence. Finally, the immobilized biotin column was connected and free Fab-AP was bound to the affinity column. The fluorescence decreased to almost the background level before adding Fab-AP, which means that virtually all Fab-AP is trapped by the immobilized biotin column. The binding efficiency was 98.5%. The binding efficiency of the immobilized biotin column for S-AP (concentration 3 U/l) was somewhat lower and was estimated at 96%. The implementation of a longer immobilized biotin column (2×20 mm) did not improved the bindings efficiencies. From these results it can be concluded that Fab-AP as well as S-AP still contains some impurities, although both affinity proteins were immuno-purified by the manufacturer.

However, the binding efficiency for both affinity proteins was sufficiently high to use the affinity proteins without further purification. The concentration of the affinity protein and the capacity of the immobilized biotin column determine the operation time before regeneration is required.

3.4. Enzyme-labeled affinity protein concentrations

In principle, the concentration of affinity proteins in enzyme assays should be as high as possible to ensure that 100% of the analyte will bind to the affinity protein. Moreover, a high concentration of affinity protein will result in a large linear range.

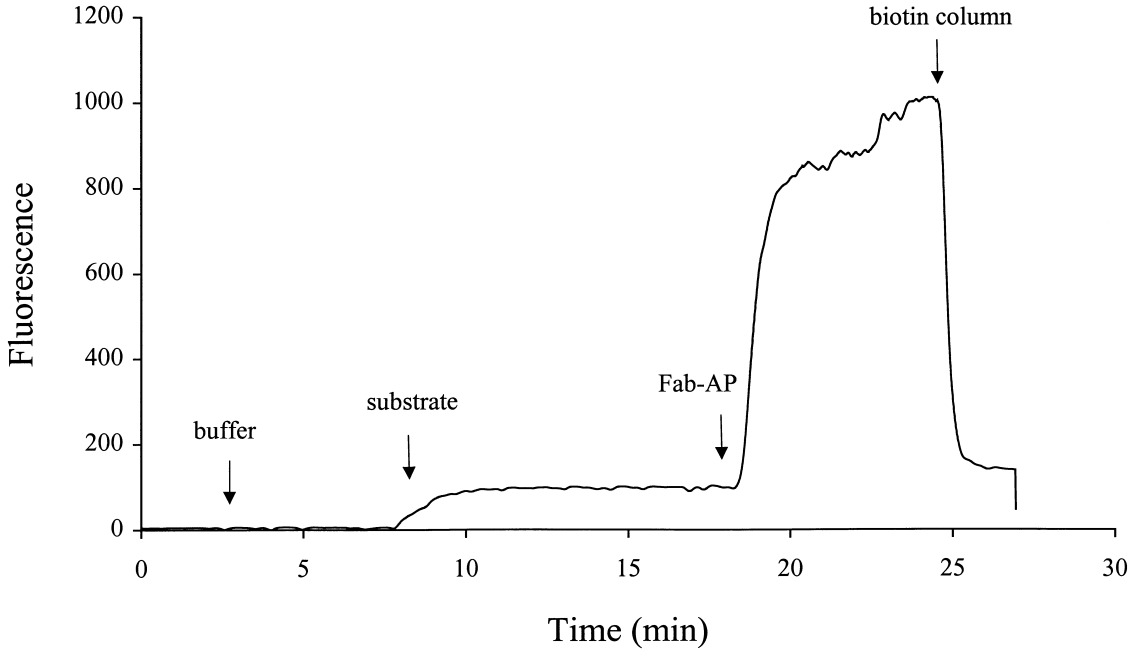

Fig. 2. Efficiency test of the immobilized biotin column. For conditions, see text.

However, it is necessary to separate the excess affinity protein from the biotin-bound fraction. In the described system, the separation is performed with an immobilized biotin column, which trapped the unbound affinity protein whereas the biotin-bound fraction passes the trapping column unretained. As mentioned previously, impurities caused an increase of the background depending on the affinity protein used. Consequently, increasing the concentration of affinity protein will increase the background. Furthermore, a high concentration affinity protein will lead to a short operation time of the immobilized biotin column. Since a small immobilized biotin column was chosen to minimize the peak broadening, the capacity of this column is rather low. Therefore, the concentration of enzyme-labeled affinity protein should be a compromise between signal, linear range, background and operation time of the immobilized biotin column.

An additional problem is the multivalency of streptavidin, which possesses four high affinity binding sites for biotin. In principle, a biotin–streptavidin mixture will be a mixture of 1:1, 1:2, 1:3 and 1:4 biotin–streptavidin complexes. However, with an excess of streptavidin mainly 1:1 complexes are formed [17], which means that there are three binding sites not occupied. These remaining binding sites allow the 1:1 complexes to bind to the immobilized biotin column.

The optimum concentration of enzyme-labeled affinity proteins was examined by injection of a fixed amount of 50 fmol BACH. The EA-BCD system (Fig. 1) was used to investigate five S-AP concentrations, ranging from 1.0 to 5.0 U/l.

After the on-line incubation, the excess of affinity protein was separated from the biotin-bound affinity protein by the immobilized biotin column, followed by substrate addition. The S/N alteration observed when BACH ($n=6$) was injected with different S-AP concentrations is given in Fig. 3a. The S/N increased when the S-AP concentration increased from 1.0 to 3.0 U/l. A further increase of the S-AP concentration to 5.0 U/l lead to a further increase of the signal but to a decrease of the S/N ratio because of a higher noise. From these results, it can be concluded that the incubation of biotin with the affinity protein is not complete when a low S-AP concentration is applied. However, with a higher concentration S-AP, the background was also increased caused by impurities, which have no affinity to the immobilized

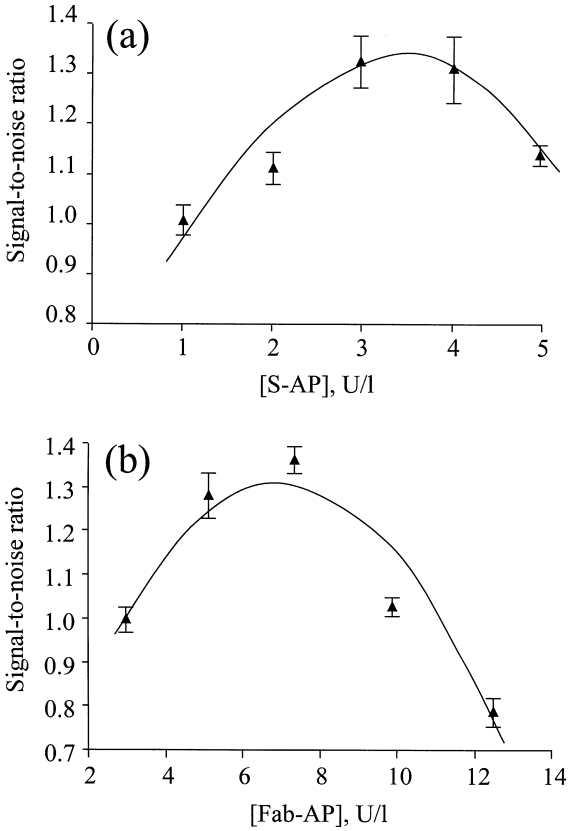

Fig. 3. Influence of the affinity protein concentration on the signal-to-noise ratio. (a) Signal obtained when S-AP was used as affinity protein, (b) signal obtained when Fab-AP was applied as affinity protein. Values are related to the signal obtained at the lowest concentration used. The error bars represent the standard deviation ($n=6$).

biotin column. Therefore, 3.0 U/l was used with further experiments. With an S-AP concentration of 3.0 U/l, the immobilized biotin column can be used for 500 injections (analysis time, 5 min) before saturation occurs. From these results it can be calculated that the immobilized biotin column has a capacity of trapping 7 μg S-AP.

The same set-up was used to investigate five different Fab-AP concentrations, ranging from 3.0 to 12.5 U/l. The S/N of BACH ($n=6$) obtained with these five concentrations is shown in Fig. 3b. The S/N increased when the Fab-AP concentration was increased from 3.0 to 7.5 U/l. The signal was even further increased with a concentration of 12.5 U/l. However, at this concentration breakthrough of Fab-AP impurities lead to a higher noise and therefore to a decreased S/N. Using a concentration of 7.5 U/l in further experiments, which give the highest S/N, the immobilized biotin column could be used for 200 injections (every 5 min) without replacement or regeneration. The immobilized biotin material is stable for at least six months when stored in the refrigerator.

3.5. Enzyme–substrate reaction temperature

Enzyme–substrate reactions are influenced by temperature, depending on the optimum working temperature of the enzyme. The optimum working temperature of alkaline phosphatase is estimated at 37°C [20,21]. At a higher temperature, the enzyme activity will be higher although finally denaturation of the enzyme occurs, which will decrease the substrate conversion dramatically. This denaturation of the enzyme caused by high temperature is also time dependent.

Because in flow systems the enzyme–substrate reaction takes only a few minutes, a higher temperature during a short period of time can increase the total substrate conversion.

To determine to optimum temperature the enzyme–substrate reaction was monitored at ambient temperature, 37, 50, 60 and 70°C. The EA-BCD system was used without the immobilized biotin column and with an S-AP concentration of 1 U/l. Omitting the immobilized biotin column will lead to a continuous substrate conversion by the enzyme label. The enzyme activity was monitored with fluorescence detection and is represented in Fig. 4. From this figure it can be concluded that an increase of the temperature induces a higher enzyme activity, which result in a higher signal. However, at 60°C the noise also increases. When the temperature was further increased to 70°C the signal dropped dramatically caused by denaturation of the enzyme. Moreover, at high temperatures, methanol from the FIA effluent starts to evaporate which lead to a higher noise. The highest S/N was found at 50°C, which was used in further experiments.

3.6. Incubation time

The flow-rate and the volume of reaction coil 1

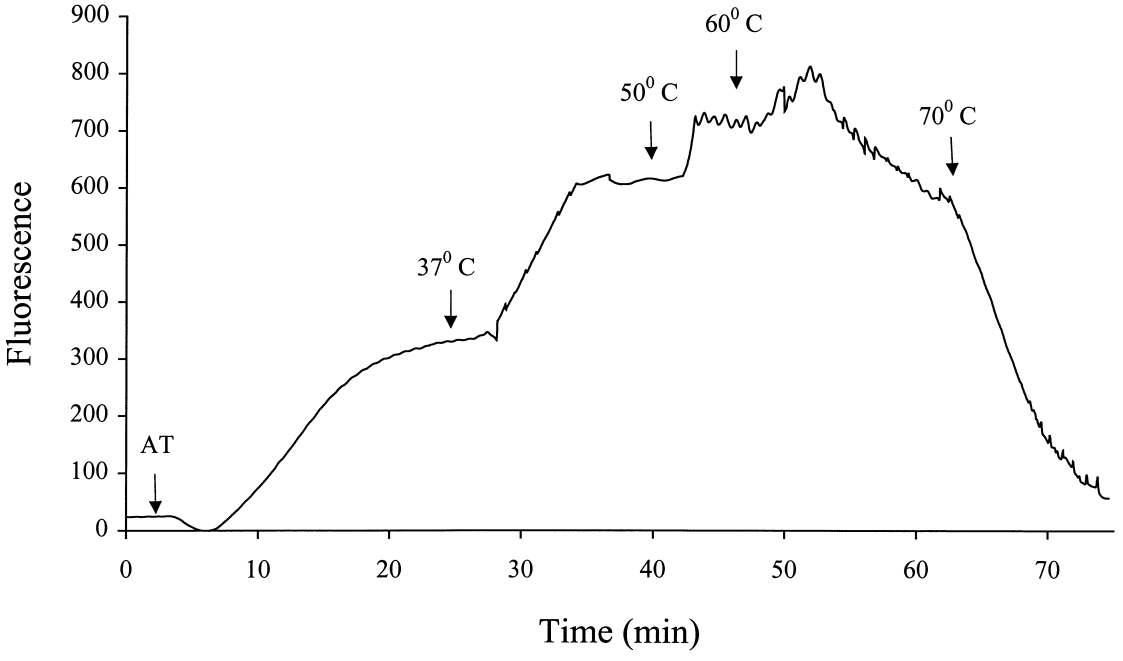

Fig. 4. Influence of the temperature on the enzyme–substrate reaction. AT=ambient temperature; for conditions, see text.

determine the incubation time of BACH with enzyme-labeled affinity proteins. Ideally, BACH is completely associated with affinity protein before transferred to the immobilized biotin column. Therefore, the incubation time should be long enough for a total association.

However, even in a perfectly knitted reaction coil, dispersion of the analytes occurs [17]. Consequently, increasing the incubation time will result in peak broadening and therefore in a lower S/N.

Reaction coils with a volume of 175, 278, 351, 571 and 745 μl (250 μm I.D.), resulting in incubation times from 48 to 203 s, respectively, were examined with the EA-BCD system. The volume of reaction coil 2 was 955 μl (500 μm I.D.). A fixed amount of 50 fmol BACH ($n=5$) was injected and incubated on-line with S-AP. Excess S-AP was removed by means of an immobilized biotin column. The influence of the incubation time on the peak area is presented in Fig. 5. With a total flow-rate of 220 μl/min through coil 1, an optimum was found with the 745 μl reaction coil.

A smaller S/N was observed with smaller reaction coils, caused by incomplete association of BACH with S-AP. However, with the 571 μl and the 745 μl reaction coils the peak-broadening increases with 15% compared to the smaller reaction coils. For shorter analysis time and to avoid peak broadening, the 351 μl reaction coil was applied in further experiments.

In enzyme assays, the signal amplification is proportional to the enzyme–substrate reaction time. Analogous to the incubation time in the first reaction coil, the flow-rate and the volume of the second reaction coil determine the enzyme–substrate reaction time. A longer reaction time will result in a higher signal but will also lead dispersion of the analytes in the reaction coil. Therefore, the volume of the second reaction coil should also be a compromise between reaction time and peak broadening.

Reaction coils with a volume of 278, 351, 571, 745 μl (250 μm I.D.), 751 and 955 μl (500 μm I.D.) were examined by injection of 50 fmol BACH. The influence of the volume of the reaction coil, and therefore the influence of the reaction time, on the signal is presented in Fig. 6. Peak area appeared to be proportional to the enzyme–substrate reaction time. Unfortunately, the backpressure of the 745 μl

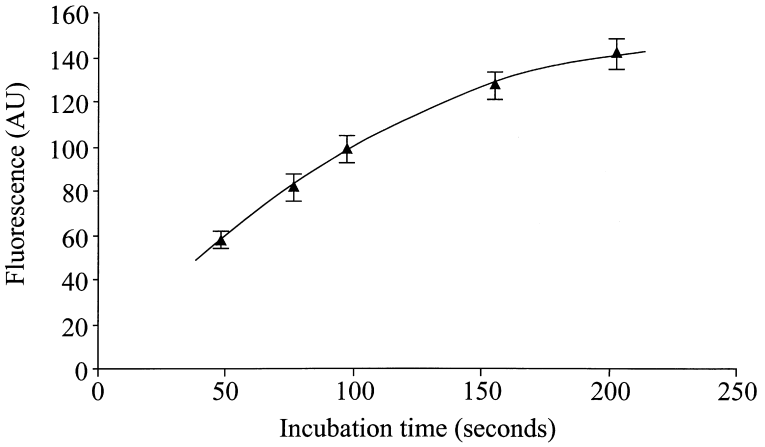

Fig. 5. Influence of the incubation time on the peak area obtained when 50 fmol BACH was injected. The error bars represent the standard deviation (n=5). For conditions, see text.

coil was too high for the peristaltic pump at the flow-rates used leading to erroneous results. Therefore, the results obtained with the 745 μl coil were omitted. Finally, a 751 μl coil and a 955 μl coil, both with an I.D. of 500 μm were tested. The larger I.D. gives a lower backpressure, which was compatible with the low-pressure peristaltic pump. The use of reaction coils with a wider I.D. leads to an increase in peak broadening of 24%. However, the 955 μl coil lead to a reaction time of 185 s with the flow-rates applied, which give the highest S/N.

3.7. Investigation of different biotinylation reagents

The interaction of the affinity protein with biotin containing compounds takes place via the bicyclic biotin moiety [12,13]. Therefore, reactive probes or analytes attached to the valeric acid side chain should have no influence on the affinity interaction. Theoretically, all biotin-containing compounds should be detected by EA-BCD with the same sensitivity, resulting in a LOD independent of the primary analyte structure. To investigate the re-

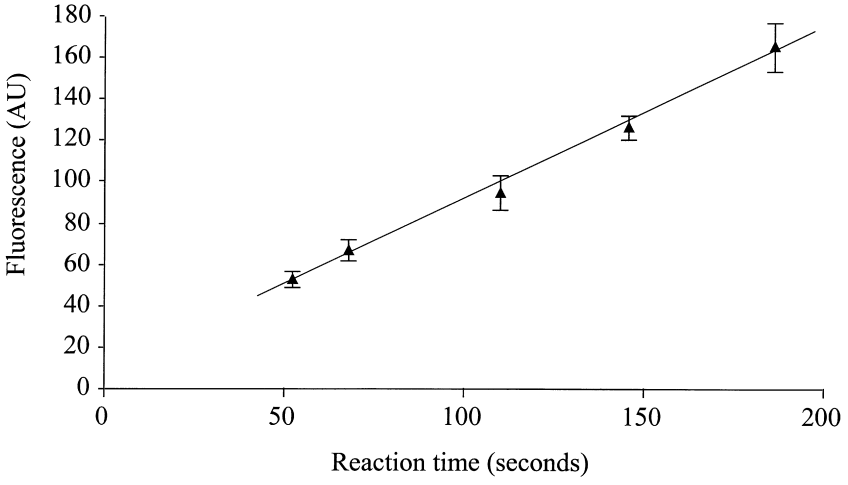

Fig. 6. Influence of the enzyme–substrate reaction time on the peak area. The error bars represent the standard deviation (n=5). For conditions, see text.

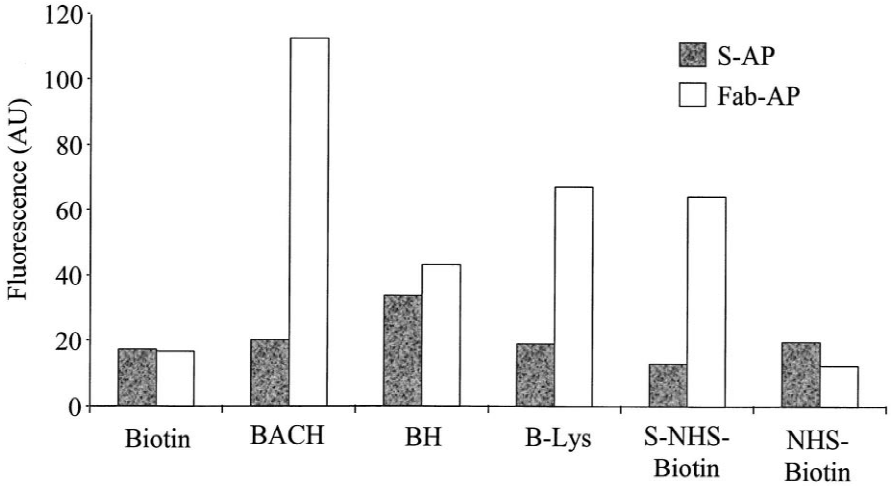

Fig. 7. Influence of biotin derivatives on the EA-BCD response. For conditions, see text.

sponse of different biotinylation reagents the LOD of biotin, BACH, BH, B-lys, S-NHS-biotin and NHS-biotin were determined with FIA-EA-BCD. The biotin-containing compounds were diluted to a final concentration of $1 \cdot 10^{-8}$ mol/l and 1 μl was injected ($n=5$). S-AP and Fab-AP were both examined. Fig. 7 shows the peak area obtained for the different biotinylation reagents. When S-AP was applied as affinity protein, the peak areas for all biotin-containing compounds were approximately the same. The influence on the interaction for different biotin derivatives with streptavidin was rather small. The

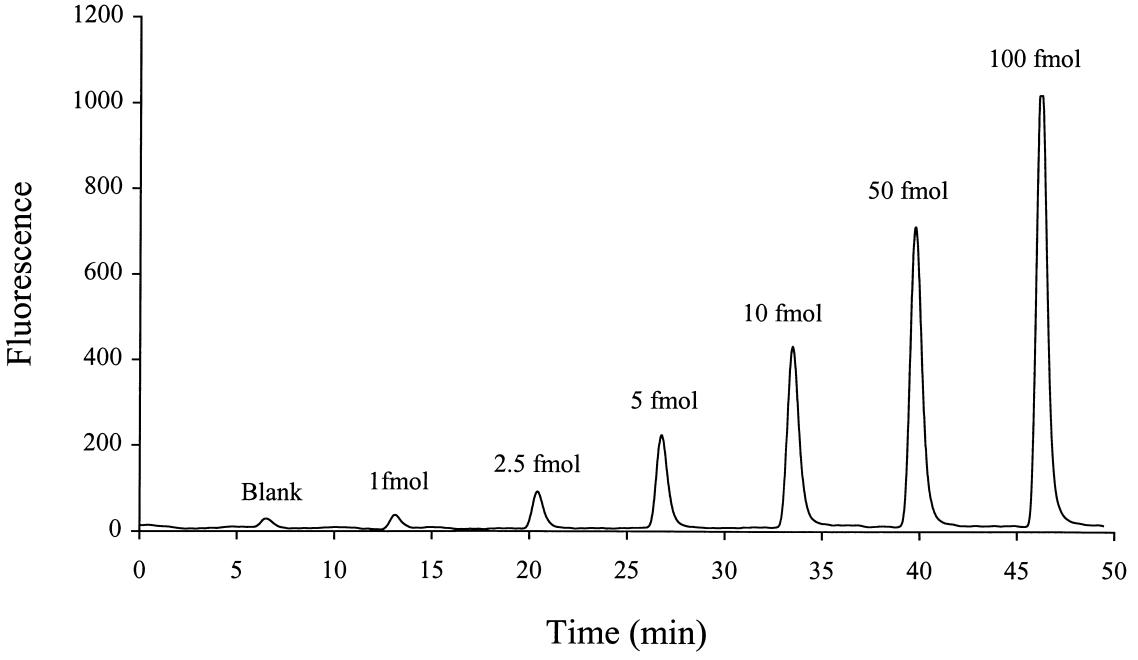

Fig. 8. Flow injection analysis of BACH, injection every 6 min. Fab-AP was used as affinity protein. For conditions, see text.

highest LOD was obtained for S-NHS-biotin, 4 fmol whereas the lowest LOD was determined for BH, 1.5 fmol.

A larger effect on the response of biotin derivatives was found when Fab-AP was applied as affinity protein. The lowest LOD of 1 fmol was observed for BACH. The LOD determined for NHS-biotin was 10 fmol. Remarkable is that the lowest LODs were found for biotin derivatives which contains a spacer between the biotin moiety and the reactive probe, BACH, B-lys and S-NHS-biotin. An explanation for this observation is that the anti-biotin Fab fragments are raised against biotin compounds which contains a spacer, rather than biotin itself. An important subject for future studies is the response of a biotinylated analyte for both affinity proteins.

3.8. Quantitative aspects

The EA-BCD system coupled to FIA was investigated with both Fab-AP and S-AP. Calibration curves were obtained by injection of 1 μl BACH dissolved in methanol–water (10:90) (Fig. 8). Using Fab-AP as affinity protein, a linear range was obtained from $2.5 \cdot 10^{-9}$ mol/l to $1.0 \cdot 10^{-7}$ mol/l with a correlation coefficient of 0.994 ($n=4$). The calibration plot was characterized by $y=5.0 \cdot 10^{9}x+19.06$ (x=concentration BACH, y=peak area). The relative standard deviation was below 4.3% for all data points within the linear range. A LOD of 1 fmol was achieved ($S/N=3$). When S-AP was applied as affinity protein a somewhat higher LOD of 2.5 fmol was obtained. Linearity ranged from $2.5 \cdot 10^{-9}$ mol/l to $2.0 \cdot 10^{-7}$ mol/l with a correlation coefficient of 0.991 ($n=4$). The calibration plot with S-AP was characterized by $y=1.77 \cdot 10^{9}x-13.16$ (x= concentration BACH, y=peak area). For all data points within the linear range, the relative standard deviation was below 5.3%.

4. Conclusions

A sensitive EA-BCD system for the detection of biotin containing compounds was developed. The system was optimized for several parameters, such as substrate, reaction time and reaction temperature. The most sensitive substrate for the fluorescence detection of alkaline phosphatase appeared to be Attophos, with an LOD for alkaline phosphatase of 8 amol. S-AP and Fab-AP were both investigated as affinity proteins. The lower linear range limit for both affinity proteins is 2.5 fmol. Therefore, both affinity proteins can be used with approximately the same sensitivity.

The coupling of HPLC to EA-BCD after pre-column biotinylation is presented in a subsequent paper [16]. The influence of the HPLC mobile phase on the interaction of biotin with the affinity proteins will be examined. Another important subject will be the labeling of analytes with biotin at low concentration level. Biotinylation is widely used to label analytes. Because the EA-BCD system respond to biotin, no adaptation of this system has to be made when different analytes are labeled with biotin. However, the affinity of biotin labeled compounds to the affinity proteins has to be examined.

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