

Preferential labeling of α -amino N-terminal groups in peptides by biotin: application to the detection of specific anti-peptide antibodies by enzyme immunoassays

I. Sélo ^{a,*}, L. Négroni ^a, C. Créminon ^b, J. Grassi ^b, J.M. Wal ^a

^a Laboratoire Associé INRA-CEA d'Immuno Allergie Alimentaire, SPI Bât 136, CE-Saclay, 91191 Gif sur Yvette Cedex, France

^b CEA, Service de Pharmacologie et d'Immunologie, DRM, CE-Saclay, 91191 Gif sur Yvette Cedex, France

Received 24 April 1996; revised 4 July 1996; accepted 26 August 1996

Abstract

Experimental conditions (pH 6.5, 24 h reaction, peptide: biotin ratio 1:5) were defined for preferential incorporation of the biotin molecule in the N-terminal α -amino group of peptides. This strategy could be helpful in numerous applications when an entire peptide chain must remain accessible for antibody or receptor binding. We illustrate this advantage in a solid-phase enzyme immunoassay designed to detect antibodies specific for bovine β -lactoglobulin present in rabbit or human sera. This test involves synthetic peptides biotinylated in different positions and immobilized on a solid phase. The use of biotin/streptavidin interactions permitted more efficient detection of specific anti-peptide antibodies than solid phases prepared using conventional passive-adsorption techniques. The highest levels of antibody binding were measured when biotinylation occurred at the N-terminal extremity of immobilized peptides.

Keywords: Biotin-(strept)avidin; Tryptic peptide immobilization; Enzyme immunoassay; Bovine β -lactoglobulin

1. Introduction

Avidin-biotin techniques have been widely used to label peptides or proteins in biochemical, immunocytochemical and immunoanalytical studies

Abbreviations: AChE, acetylcholinesterase; Blg, bovine β -lactoglobulin; DMF, dimethyl formaldehyde; EIA, enzyme immunoassay; HSA, human serum albumin; NHS-LC-biotin, sulfo-succinimidyl 6-(biotinamido) hexanoate; PBS, phosphate buffered saline; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +33.1.69.08.52.64; Fax: +33.1.69.08.59.07.

(Guesdon et al., 1979; Wilchek and Bayer, 1990; Schwartz et al., 1991; Ahmed et al., 1992; Berghorn et al., 1994). In the field of immunoassays, it is imperative to use effective and reliable coating procedures when developing specific tests suitable for research as well as for diagnostic purposes (Voller, 1975; Geerligs et al., 1988; Cesbron-Delauw et al., 1992; Coursaget et al., 1994; Gruen et al., 1994; Roy et al., 1994). Currently, coating is achieved by passive adsorption of antigens or antibodies to a solid phase using techniques described since the end of the sixties (Catt and Tregear, 1967). For instance, the first competitive enzyme immunoassays (Engvall and Perlmann, 1971, 1972) involved the reaction of im-

mune serum with passively adsorbed antigens on polystyrene tubes. Subsequent papers dealt with the measurement of various antigens and proposed several modifications of the coating procedure (Avrameas and Guilbert, 1972; Belanger et al., 1973; Hoffman, 1973; Yorde et al., 1976; Butler et al., 1978; Ishikawa et al., 1990; Hashida et al., 1991). Passive coating to the plastic support takes advantage of the hydrophobic interactions of the molecules with the polymer surface. However, in some situations, problems arise from the fact that the antigen, especially small peptides, can be released during the immunoreaction (Engvall et al., 1971). Furthermore, adsorbed proteins (antigens or antibodies) may undergo denaturation or conformational changes (Bull, 1956; Suter and Butler, 1986; Suter et al., 1989; Dierks et al., 1986; Butler et al., 1992), possibly leading to significant modifications of their immunoreactivity. For example, monoclonal antibodies immobilized by passive adsorption were shown in one study (Butler et al., 1992) to lose 90–95% of their binding capacity, the remaining binding sites exhibiting a reduced affinity for the antigen. These problems, inherent to passive coating procedures, may be solved by covalent coupling to the solid phase. This approach renders the preparation of the solid phase much more complicated and does not always provide the most effective coating (Boudet et al., 1991). The use of biotinylated molecules has been described as an interesting alternative procedure. Here, biotinylated proteins are immobilized through interactions with coated avidin conjugates. The efficiency of the coating is ensured by the extraordinary affinity ($K_a = 10^{15} \text{ M}^{-1}$) of the biotin for the (strept-)avidin.

Except for synthetic peptides (Fisher and Howden, 1990; Ivanov et al., 1992), which are easily biotinylated selectively at the α N-terminal group during their synthesis, it is not easy to control either the number of biotin molecules incorporated in the peptide or the proteins, or their positions along the peptide chain. In fact, most of the techniques using biotinylated peptides have involved small peptides without any lysine residue along the peptide chain, or only one residue located near the N-terminal extremity (Ishikawa et al., 1990; Anumula et al., 1990; Hashida et al., 1991). As already pointed out by Gaudriault and Vincent (1992), in relation to the Bolton-Hunter reagent, selective labeling of α - or

ϵ -amino groups in peptides could be a critical factor, especially when small peptides have to be biotinylated, because coupling the ϵ -amino groups of various lysine residues with a biotin molecule could greatly alter the ability of the peptide to be recognized by an antibody in an immunological test.

In the present paper, we describe experimental conditions permitting preferential coupling of biotin to the α -amino group of the N-terminal amino acid. The two synthetic peptides used in this study were derived from the sequence of bovine β -lactoglobulin (Blg), a protein thought to be involved in milk allergy (Goldman et al., 1963; Bleuminck and Young, 1968; Wal et al., 1995). We show that peptides selectively coupled to biotin via their N-terminal amino group are particularly suited to the detection of specific antibodies both in the serum of a rabbit immunized with Blg and in human sera from patients allergic to cow's milk. We suggest that this methodology, which preserves most of the ϵ -amino groups on biotinylated peptides, could be of special interest in experimental protocols for biochemical or immunocytochemical studies involving peptide interactions with an antibody or a biological receptor.

2. Materials and methods

2.1. Peptide synthesis and characterization

Two peptides were designed for use in this study: KGLDIQKVAGTWYSLA ((8–23) peptide), and IQKVAGTWYSLA ((12–23) peptide). They were synthesized using *t*-BOC chemistry on a solid-phase peptide synthesizer (9050 Milligen). Characterization was achieved using HPLC, amino acid analysis and mass spectrometry. Peptide purity was > 95%. Another peptide, unrelated to Blg (Lox1 peptide: RKSSGFPSVKSREQLSGY) was used in control experiments.

2.2. Biotinylation of peptides

Using the procedure of Diamandis and Christopoulos (1991), biotinylation was performed using NHS-LC-biotin (Pierce) possessing an extra spacer arm thereby rendering the biotin molecule available for avidin or streptavidin binding.

In a first series of experiments, (12–23) peptide (70 nmol) was dissolved in phosphate buffer (120 μ l at 50 mM) at different pHs (6.5, 7.5, 8.5) before addition of 700 nmol of NHS-LC-biotin dissolved in 20 μ l DMF. The reaction was carried out at 4°C for 120 min and stopped by acidification with 80 μ l of 1% TFA.

In a second set of experiments, different amounts of NHS-LC-biotin (80, 200, 400 nmol in DMF (11 μ l)) were allowed to react with the same amount of (12–23) peptide (40 nmol at pH 6.5). The reaction was carried out at 4°C for 1 to 24 h and stopped as already described.

2.3. Peptide separation and identification

2.3.1. HPLC procedure

RP-HPLC was performed using a Waters Assoc. chromatograph, equipped with two M 510 pumps and a Wisp 712 automatic injector. Samples were applied to a Nucleosil C18 RP-HPLC analytical column (C18, 5 μ m, 10 nm, 4.6 $\times$ 250 mm, SFCC) and elution was achieved with a linear gradient from 25 to 50% of solvent B (100% acetonitrile 0.04% TFA) in solvent A (0.1% TFA) over 25 min at a 1 ml/min flow rate. Absorbances were monitored at 280 nm using an SPD-10A UV-VIS detector (Shimadzu). Once collected, all fractions were dried under vacuum with a Speedvac SVC 200 (SAVANT) and stored at –20°C.

2.3.2. Biotin enzyme assay

The strategy of the enzyme assay of biotin was based on the competition between solutions of variable biotin concentrations and biotinylated-HSA pre-coated on microtiter plates for streptavidin-AChE (acetylcholinesterase) binding. A supplementary step to the assay described by Chang et al. (1994) was introduced as an indispensable alteration used in order to prevent any steric hindrance of the avidin-biotin interaction. Biotinylated peptides were digested overnight at room temperature by 1% pronase protease (type VI from *Streptomyces griseus*, Sigma) in PBS using an enzyme:substrate ratio = 1:100. After heating at 100°C for 2 min, the solutions were dried.

The competitive enzyme assay used to measure free biotin involved the AChE-streptavidin conjugate (streptavidin from *Streptomyces avidinii*, Sigma) as tracer (according to Frobert and Grassi, 1992) and microtiter plates (96F immunoplates certificate from NUNC) passively coated with a biotin-HSA conjugate (150 μ l/well at 5 μ g/ml for 24 h at room temperature). HSA (from Centre National de Transfusion Sanguine) was biotinylated according to the manufacturer's standard conditions (Pierce). After five washes with 0.1 M phosphate buffer pH 7.5 containing 0.05% Tween 20, using a microplate washer 120 (Labsystem), the microtiter plates were filled using an automatic dispenser (autodrop from Labsystem) with 300 μ l of 2% polyvinyl pyrrolidone (wt 40 000, Sigma) in PBS buffer pH 7.2 (Biom  rieux). Plates were stored at 4°C and rinsed five times before use. Different dilutions of protease-treated biotinylated peptides or of standard biotin in EIA buffer (0.1 M phosphate buffer pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.01% sodium azide, 0.1% HSA) were incubated with gentle stirring for 2 h at room temperature with the streptavidin-AChE conjugate (used at 1 Ellman's unit/ml as defined by Pradelles et al. (1985)). Prereacted samples were distributed into microtiter plates coated with biotinylated HSA and agitated for 2 h at room temperature. After washing, AChE activity retained on the plate was measured by Ellman's method (Ellman et al., 1961). After adding Ellman's reagent (200 μ l/well), absorbance was recorded at 414 nm with a multiskan spectrophotometer (Labsystem). Biotin concentration in the samples was determined by reference to the standard curve obtained using known concentrations of NHS-LC-biotin.

2.3.3. Trypsin hydrolysis and RP-HPLC analysis of biotinylated peptides

A 10 μ l aliquot of a 2 μ g/ml trypsin solution (trypsin EC 3.4.21.4 TPCK-treated) in 25 mM phosphate buffer pH 7.5 was added to 150 μ l of biotinylated peptides. This corresponds to an enzyme:substrate ratio of 1:250 (w/w). After 1 h of incubation at room temperature, the reaction was stopped by the addition of 1% TFA (70 μ l). Reaction mixtures were applied to the C18 analytical column and analyzed using the conditions described above.

2.4. EIA procedures for detecting specific antibodies in serum

2.4.1. Coating of biotinylated and unbiotinylated peptides

Biotinylated peptides were immobilized on microtiter plates via interactions with an HSA-biotin conjugate using streptavidin as a linker. The biotinylated HSA was passively adsorbed on the plate using similar conditions to those described above in the biotin assay. After washing, streptavidin (20 $\mu\text{g}/\text{ml}$) was added to each well (50 $\mu\text{l}/\text{well}$) and plates were gently agitated for 4 h at room temperature. After washing, biotinylated peptides (50 $\mu\text{l}/\text{well}$ at 0.5 nmol/ml in EIA buffer) were added and plates were left for at least 4 h at room temperature with gentle agitation.

Using the conventional methodology (Catt and Tregear, 1967), non-biotinylated peptides were directly coated on the solid phase (75 $\mu\text{l}/\text{well}$ at 10 nmol/ml peptides in 50 mM carbonate buffer pH 9.0 for 24 h at room temperature). After washing, each well was filled with the saturation buffer (HSA buffer). Plates were kept overnight at 4°C before use.

2.4.2. EIA measurement of anti-peptide antibodies

2.4.2.1. Rabbit anti-Blg antiserum. After washing, plates were first filled with various dilutions of a specific rabbit anti-Blg antiserum (50 $\mu\text{l}/\text{well}$ in EIA buffer), then washed after an 18 h reaction at 4°C and finally filled with 50 μl of an anti-rabbit Ig mouse monoclonal antibody (Metreau et al., 1987) labeled with AChE, as described by Grassi et al. (1989). After a further 4 h reaction at room temperature and a washing step, solid phase-bound AChE activity was measured as previously described.

2.4.2.2. Human sera from allergic and non-allergic patients. As for the rabbit antiserum, plates were first filled with various dilutions of human sera (50 $\mu\text{l}/\text{well}$ in EIA buffer), then washed after a 24 h incubation at room temperature and finally filled with 50 μl of an anti-human IgE monoclonal antibody (BS17 described by Grassi et al., 1986) labeled with AChE. After a further 18 h at room temperature, AChE activity was measured as described above.

Specific IgE to whole Blg protein present in plasma samples A₁, A₂ and A₃ was assayed as described by Wal et al. (1995). The respective values were 20, 229, and 26 IU/ml.

3. Results

3.1. Direct identification of biotinylated peptides using standard biotinylation conditions

The (12–23) synthetic peptide, whose sequence is derived from bovine β -lactoglobulin (Blg), was chosen as a model peptide since it contains only one ε -amino group (Lys¹⁴) in addition to the α -amino terminal group.

Fig. 1 shows the HPLC elution profile of the products obtained by reacting the (12–23) peptide with biotin under standard conditions i.e. at pH 8.5 for 1 h, with a peptide:biotin ratio of 1:50. Four different peaks were detected by absorption at 280 nm. Biotinylated products were identified by precisely measuring the amount of biotin present in each peak through a competitive enzyme assay, and by assessing the resistance of the peptide present in each peak to trypsin endopeptidase.

3.1.1. Determination of the biotin / peptide ratio

The free biotin, released from peptides through pronase hydrolysis (see Section 2), was quantified by

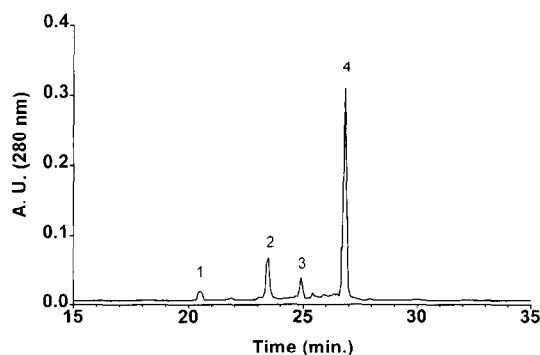


Fig. 1. HPLC analysis of products obtained by biotinylation of the (12–23) peptide using standard conditions (at pH 8.5, for 1 h with a peptide:biotin ratio of 1:50). HPLC elution was carried out as described in Section 2. Key: peak 1, non-biotinylated peptide; peak 2, Lys¹⁴ ε -amino biotinylated peptide; peak 3, α -amino N-terminal biotinylated peptide; peak 4, di-biotinylated peptide.

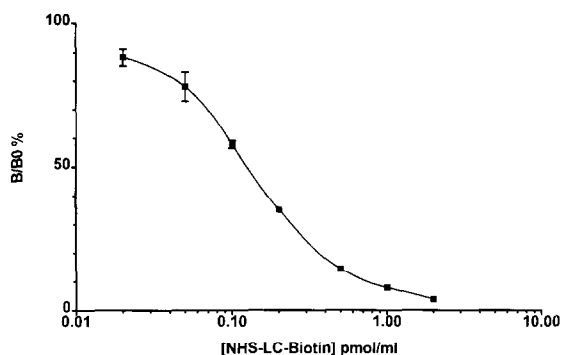


Fig. 2. Standard curve obtained with the competitive enzyme assay of biotin. Values in duplicate are expressed as a percentage $\pm$ SD of the binding values measured in the absence of NHS-LC-biotin (B_0).

reference to a standard inhibition curve (Fig. 2). The sensitivity of this standard biotin curve for the measurement of 50% inhibition was calculated as $0.15 \text{ pmol/ml} \pm 0.03$ ($n = 14$ assays). Similar values were obtained using NHS-LC-biotin or Gly-LC-biotin as competitor (data not shown).

Since the amount of peptide present in the samples was readily derived from the optical density measurement at 280 nm, the biotin enzyme assay allowed of the biotin:peptide ratio to be calculated for the individual peptides present in each one of the four peaks revealed by RP-HPLC analysis after biotinylation of the (12–23) peptide. This ratio was found to be 0.1 ± 0.05 , 1 ± 0.2 , 0.9 ± 0.1 and 2.1 ± 0.3 (mean $\pm$ SD of nine determinations) for the peptides in peaks 1, 2, 3 and 4, respectively.

3.1.2. Identification of mono- and di-biotinylated peptides

Biotin:peptide ratio determinations indicated that peaks 1 and 4 corresponded to non-biotinylated and di-biotinylated peptides, respectively, while mono-biotinylated peptides (either in the N-terminal or Lys¹⁴ position) were recovered in peaks 2 and 3. Trypsin was not able to hydrolyze mono-biotinylated peptides from peak 2, as deduced from the single peak recovered in HPLC after enzymatic treatment. Since coupling at the ϵ -position of the Lys¹⁴ side chain prevents hydrolysis, it was concluded that peptides eluted in peak 2 were biotinylated exclusively in that position. By contrast, peptides from peak 3 were recovered in three peaks after trypsin

hydrolysis, corresponding to the two digestion products in addition to a small amount of non-hydrolyzed peptide (data not shown). Thus, the native peak 3 corresponded to the peptide which had been selectively labeled by biotin in the α -amino N-terminal position.

3.2. Optimization of the biotinylation procedures to enhance labeling of the α -amino N-terminal group

Not surprisingly, conventional biotinylation conditions (basic pH and high biotin/peptide ratio) favored extensive labeling of the peptide, with a prominent peak 4 (di-biotinylated peptide) and a much reduced peak 1 (non-biotinylated peptide). Since, under these conditions, the α -biotinylated peptide (peak 3) represented only about 11% of the total biotinylated peptides recovered after the biotinylation reaction (Fig. 1), our experimental protocols focused on the possibility of increasing this relative proportion.

3.2.1. Effect of variation in the pH

Using a biotinylation reaction time of 2 h and a constant biotin:peptide ratio of 10:1, the relative proportions of the different peaks were found to differ with pH. As shown in Fig. 3, lowering the pH of the biotinylation reaction from 8.5 to 6.5 led to a substantial increase in the proportions of peaks 2 and 3 i.e. mono-biotinylated peptides over peak 4 (di-biotinylated peptide). Peak 3, which at pH 8.5 represented about 37% of all biotinylated products, amounted to about 58% of biotinylated peptides at pH 6.5. However, pH values lower than 6.5 were not tested because the recovery rate of the biotinylated products rapidly declined when the pH was lowered, as revealed by the dramatic increase in peak 1 (non-biotinylated peptide) at pH 6.5.

At pH 6.5 (Fig. 3C), and under these biotinylation conditions, the ratios of α -amino over ϵ -amino biotinylated peptide and of α -amino over di-biotinylated peptide were close to 2:1 and 3:1, respectively.

3.2.2. Effect of variation in the biotin:peptide ratio and the biotinylation reaction time

At a biotinylation reaction pH of 6.5, three biotin:peptide ratios (10:1, 5:1, 2:1) and 5 incubation

tion times (1, 2, 3, 5, and 24 h) were tested. As seen from Table 1, the relative proportion of α -amino biotinylated peptide (peak 3) was optimal for a biotin:peptide ratio of 5:1, whatever the duration of the reaction. However, the total production of this peptide was rather slow and required a 24 h reaction. Under these conditions, the α -amino biotinylated peptide amounted to 63% of all biotinylated products (Table 1), the ratios (given by the HPLC profile -not shown-) of α -amino over ε -amino biotinylated peptide and over di-biotinylated peptide reached values of 3:1 and 6:1, respectively i.e. 6 and 60 times more than with standard biotinylation procedures (Fig. 1).

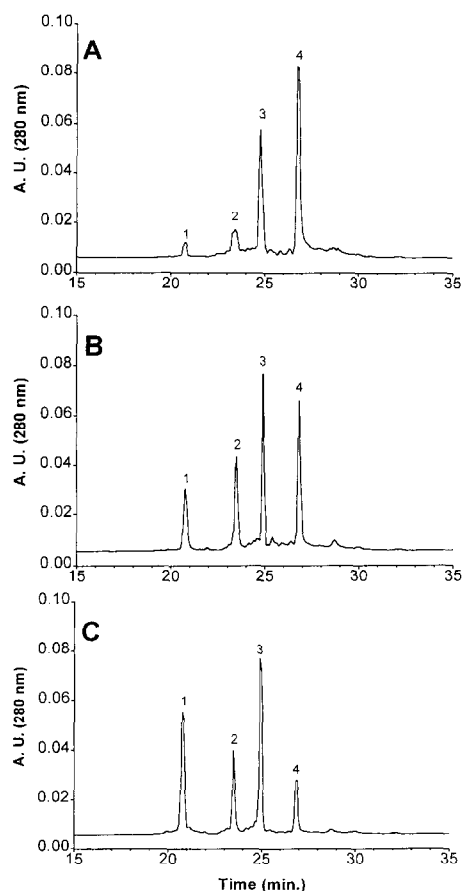


Fig. 3. HPLC analysis of products obtained by biotinylation of the (12–23) peptide for 2h, with a peptide:biotin ratio of 1:10 and at different pH values: 8.5 (A), 7.5 (B), and 6.5 (C). Incubation of peptides in the presence of biotin and HPLC elution were carried out as described in Section 2. Key: peaks 1–4 as in Fig. 1.

Table 1

Products obtained by biotinylation of the bovine β -lactoglobulin (12–23) peptide at pH 6.5 using different reaction conditions

	Biotine:peptide	Reaction time				
		1 h	2 h	3 h	5 h	24 h
A	2:1	54.1	61.6	60.0	58.4	41.0
	5:1	69.3	67.4	66.3	34.5	63.0
	10:1	62.8	59.7	60.6	57.7	42.4
B	2:1	13.3	23.4	27.4	36.4	37.2
	5:1	8.0	15.4	20.2	27.3	47.3
	10:1	8.5	14.8	20.4	25.8	37.4

Products obtained by biotinylation of the Blg (12–23) peptide at pH 6.5 using different reaction times (1–24 h) and various biotin:peptide ratios (10:1, 5:1, 2:1) were analyzed by RP-HPLC. A: values represent the percentage of α -amino biotinylated peptides over the sum of mono- and di-biotinylated peptides. B: values represent the percentage of α -amino biotinylated peptides over the sum of non- and biotinylated peptides.

3.3. Use of biotinylated peptides for coating procedures in EIA

3.3.1. Detection of specific anti-peptide antibodies in an immune anti-Blg rabbit antiserum

We used not only the (12–23) peptide but also the (8–23) peptide, which contains one α -amino and two ε -amino groups (Lys⁸ and Lys¹⁴). Both peptides were biotinylated as previously defined i.e. at pH 6.5, using a biotin:peptide ratio of 5:1 and a reaction time of 24 h. After HPLC analysis, the number of bound biotin molecules per peptide was measured as explained above. No attempt was made with the (8–23) peptide to determine the precise biotin position for either the two ε -amino mono-biotinylated or the three di-biotinylated peptides. All biotinylated peptides were then individually coated on microtiter plates through biotin/streptavidin interactions, as described above, and used for detecting specific antibodies in the serum of a rabbit immunized with Blg. As seen in Fig. 4 for both the (12–23) and (8–23) peptides, the greatest antibody binding was observed when α -amino biotinylated peptides were coated. For the (8–23) peptide, however, the coating of two other products, an ε -amino mono-biotinylated form and one of the di-biotinylated forms, led to almost identical signals. This probably results from the fact that a lysine residue is found in the N-terminal position of the (8–23) peptide. As a conse-

quence, the three N-terminal biotinylated peptides (in the α -amino and/or ϵ -amino group of Lys⁸) must be very similar in terms of peptide presentation to antibodies after the coating procedures. In contrast, the three other biotinylated products from the (8–23) peptide, which probably correspond to peptides in which biotinylation occurs at the ϵ -amino group of Lys¹⁴, were less efficiently recognized by the antibodies.

Both the (12–23) and (8–23) peptides were more immunoreactive when immobilized through avidin-biotin interaction than when coated by passive adsorption (Fig. 5). For serum dilutions lower than 1/60 000, passive adsorption failed to produce any significant antibody binding to either peptide. This lack of efficiency of direct adsorption coating for detecting antibody binding was partly compensated by increasing the serum concentration, but even at a dilution of 1/3000 the amount of antibodies recognizing the immobilized peptides was still about 2.5 times lower than when biotinylated peptides were

coated through biotin-streptavidin interactions. Interestingly, the difference in the signals between the (8–23) and the (12–23) peptides, which was observed at high serum concentrations, could correspond to the detection of specific antibodies directed to an epitope located on the (8–12) sequence.

3.3.2. Detection of specific serum antibodies from human patients allergic to cow's milk

We first investigated the presence of antibodies directed to the (12–23) peptide in sera from patients allergic to milk, and especially to Blg, using different peptide coating procedures (Fig. 6). As observed with the rabbit anti-Blg antiserum, the immobilized (12–23) peptide, which was best recognized by the antibodies from the three allergic patients, was precisely the one selectively biotinylated at the N-terminal position. The antibody binding signals appeared in each case to be significantly higher (40–200% increase) than those obtained when the unpurified whole mixture of mono- and di-biotinylated (12–23)

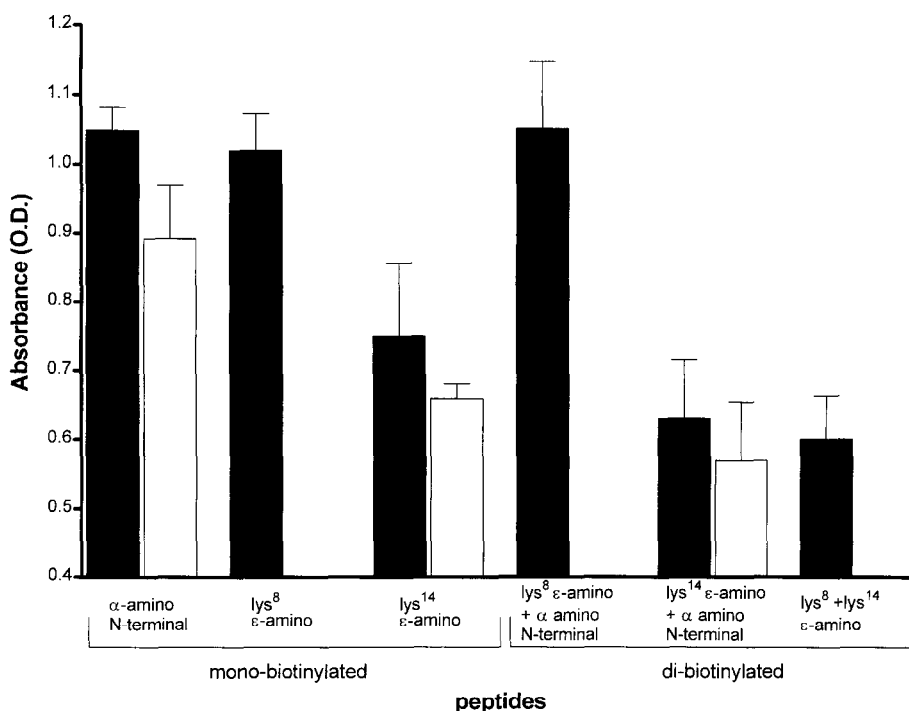


Fig. 4. Binding of anti- β -lactoglobulin rabbit antiserum to α - and/or ϵ -amino biotinylated (12–23) (white) and (8–23) peptides (black), immobilized on microtiter plates through biotin/streptavidin interactions. Values represent the mean absorbance values $\pm$ SD of four replicates tested at a serum dilution of 1/10⁴.

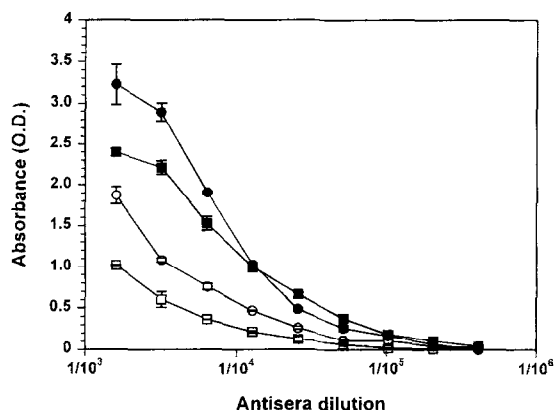


Fig. 5. Binding of anti- β -lactoglobulin rabbit antibodies to (12-23) and (8-23) peptides immobilized on microtiter plates either through biotin/streptavidin interactions after purification of the peptides biotinylated at the α -amino N-terminal position (closed symbols) or through passive adsorption (open symbols). Data represent the mean absorbance values $\pm$ SD of four replicates tested at different serum dilutions. Using a rabbit control serum from a non-immunized animal, no signal was observed ($OD < 0.07$ AU) with any immobilized peptide, or for any serum dilution (not shown). Key: $\bullet$, N-terminal biotinylated (8-23) peptide; $\circ$, (8-23) peptide; $\blacksquare$, N-terminal biotinylated (12-23) peptide; $\square$, (12-23) peptide.

peptides recovered after biotinylation under optimized conditions was used for coating. Moreover, the amount of antibody bound to the plate was 2.5–3 times higher when the peptide selectively biotinylated at the N-terminal position was immobilized through interactions with streptavidin than when it was passively adsorbed. In passive adsorption, the antibodies present in the serum of one allergic patient even failed to recognize the (12-23) peptide, emphasizing once more the advantages of coating techniques using biotin-streptavidin cross-linking.

4. Discussion

As a sulfosuccinimidyl ester derivative, biotin reacts with α - and ϵ -amino groups of peptides, strongly interacts with streptavidin, and has become a universal reagent for labeling of peptides and proteins (antibodies and enzymes). In immunoanalysis, biotinylated compounds have largely contributed to the development of sensitive enzyme immunoas-

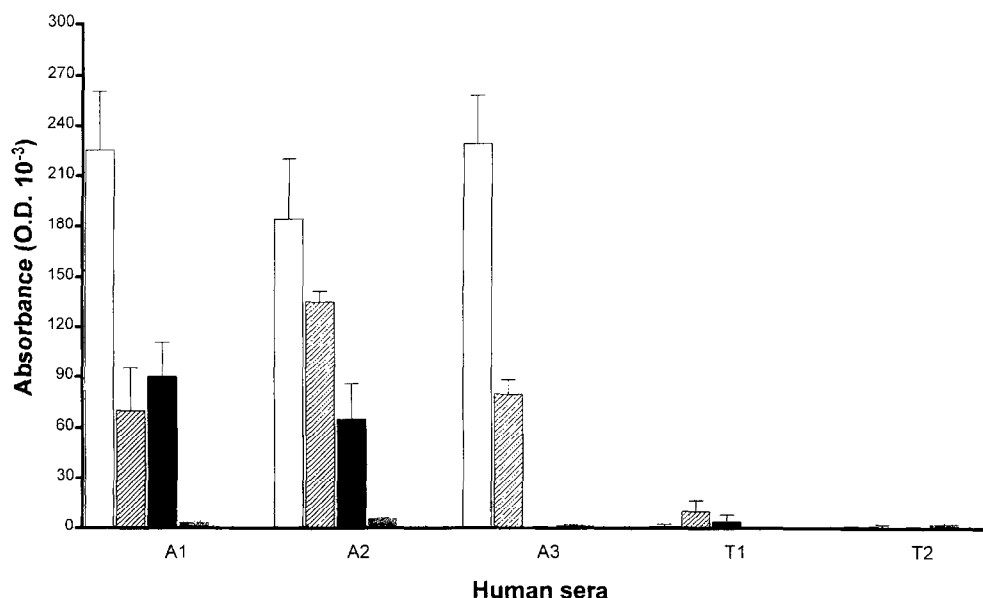


Fig. 6. Binding of IgE from human sera to (12-23) peptides immobilized on microtiter plates (1) through biotin/streptavidin interactions after purification of the peptides biotinylated at the α -amino N-terminal position (white), (2) without purification after biotinylation under optimized conditions (hatched), and (3) through passive adsorption (black). Binding of IgE from human sera to a peptide not related to Blg and immobilized on microtiter plates (1) through biotin/streptavidin interactions (dark grey), (2) through passive adsorption (light grey). Human sera A1, A2, and A3 were obtained from different allergic patients and human sera T1 and T2 were obtained from different non allergic patients. Values represent the mean absorbance values $\pm$ SD of two replicates.

says (Guesdon et al., 1979; Plebani et al., 1986; Grassi et al., 1989; Ishikawa et al., 1990; Hashida et al., 1991; Ivanov et al., 1992; Olivieri et al., 1993). The success of the biotin-avidin technique is linked to the simplicity of the biotinylation reaction, but also to the commercial availability of a large panel of enzymes and antibodies labeled either with biotin or avidin. However, a limitation of the method lies in the fact that random biotinylation of a lysine residue, especially in short peptides, can greatly alter the functional properties of the molecule or its capacity to be recognized by a binding protein, such as an antibody. In order to enhance the potential use of the biotinylation technique, we show in this paper that incorporation of a biotin molecule can be directed selectively towards the α -amino group at the N-terminal position of a short peptide, keeping almost the entire length of the molecule accessible to antibodies, as previously demonstrated by Gaudriault and Vincent (1992) using the Bolton-Hunter reagent. This can essentially be achieved by working at a pH of 6.5, i.e. significantly lower than that generally recommended for efficient biotinylation reactions. The preferential labeling of α -amino compared to the ε -amino groups at neutral or moderately acidic pH is explained by the fact that the pK_a of α -amine function ($pK_a = 8.9$) is lower than that of ε -amine function ($pK_a = 10.5$). We have also shown that it is possible to enhance further the preferential labeling of the α -amino versus ε -amino group by using a moderate biotin:peptide (i.e. 5:1) ratio and long reaction times (24 h), which compensate for the low reactivity of the amine function at pH 6.5.

As an illustration of the value of directing the incorporation of biotin towards the N-terminal amino acid in a peptide, we have shown in this study that the use of selectively biotinylated peptides for preparing an appropriately orientated solid phase significantly improved the sensitivity of enzyme immunoassay tests designed to detect anti-peptide antibodies. When N-terminal biotinylated peptides were immobilized through avidin-biotin interactions, the amount of anti-peptide antibody retained on the plate was significantly higher than when peptides biotinylated at another position were used, or when peptides were passively adsorbed on the plate. This may be simply explained by the fact that the directed biotinylation leaves the major part of the peptide ac-

cessible to antibody binding, allowing the detection of antibodies directed against epitopes which otherwise would have been either partly or totally masked by a random immobilization. For example, our coating procedure revealed the occurrence, in an anti-Blg rabbit antiserum, of antibodies directed at an epitope located around the Lys¹⁴ residue in Blg, as deduced from the difference between the signals observed using the N-terminal biotinylated (8–23) or (12–23) peptides in comparison with Lys¹⁴ ε -amino coupled peptides. Antibody binding may also be enhanced because peptides undergo less pronounced conformational changes when immobilized by biotin-avidin interactions than when simply adsorbed. In addition, when compared with passive adsorption, this method may (i) prevent any desorption of the peptides during the successive washing and incubation steps (Engvall, 1980), and (ii) avoid any variations in the capacity of small peptides to adhere to the solid phase (Geerligs et al., 1988; Ball et al., 1994).

From a quantitative point of view, the gain in sensitivity provided by the present technique for immobilizing peptides facilitates detection of antibodies in epitope mapping, when working with antisera (from allergic patients) containing low levels of specific IgE antibodies. Indeed, the signals obtained after immobilization via biotin-streptavidin interactions were always higher than those measured when passive adsorption was used. Moreover, in one case, a significant signal was measured by our coating procedure when no signal could be detected using passive adsorption. The possibility of working with highly diluted antisera has two important consequences: first, it lowers consumption of small samples (especially sera from young children), and second, it reduces artefactual interference due to the matrix effect (Plebani et al., 1986) or to the presence of heterophilic antibodies (Grassi et al., 1990; Grassi, 1994a,b).

However, care should be exercised since the peptide coating method presented here might fail to detect serum antibodies directed at the N-terminal end of the immobilized peptide. In this case, especially for short peptides, the amount of antibody retained on the plate is expected to be similar or even slightly lower than when the peptide is passively adsorbed on the plate. This is exactly what we observed when comparing the efficiency of both

coating techniques for the short (15–23) peptide (data not shown), and is in agreement with our conclusion that the rabbit anti-Blg serum contains antibodies recognizing an epitope around the Lys¹⁴ residue.

Finally, and in a more general context, the methodology described here can be used to preserve the integrity of a functionally important ϵ -amino group in a biologically active peptide. Potential applications would include the purification of the receptor of a peptide. This could be achieved using chemical cross-linking, via a homo-bifunctional reagent, to a free ϵ -amino group of the peptide, previously immobilized by its α -amino N-terminal group on a chromatographic support, through biotin-avidin interactions (Schvartz et al., 1991). It has been reported that the integrity of lysine side chains may be crucial for the binding of various neuropeptides to their biological receptors. For example, the introduction of a Bolton-Hunter moiety into the ϵ -amino groups of neurotensin or neuromedin N completely abolished the binding properties of these peptides (Gaudriault and Vincent, 1992), so it is likely that the introduction of a biotin molecule at the same position would lead to an identical effect. In studies of receptors for such ligands, the selective biotinylation method could therefore be essential not only for affinity chromatography techniques using immobilized biotinylated ligands, but also for localization microscopy using biotinylated ligands and rhodamine-labeled avidin.

In conclusion, because of the general applicability of the avidin-biotin technique, the strategy described here to orient the biotinylation of peptides towards their α -amino N-terminal group could have numerous potential applications in immunoanalysis, biochemistry and cytochemistry, for studies of the interactions between a biotinylated peptide and an antibody or a biological receptor.

Acknowledgements

This work received financial support from the European Union: programme FAIR (grant Air2-CT94-0970). The authors are greatly indebted to Professor Jean-Yves Couraud for constant interest and for helpful discussions during the course of this

work. Finally, we thank Dr. David Marsh for correcting the manuscript.

References

- Ahmed, A.R.H., Olivier, G.W.J., Adams, G., Erskine, M.E., Kinsman, R.G., Branch, S.K., Moss, S.H., Notarianni, L.J. and Pouton, C.W. (1992) Isolation and partial purification of a melanocyte-stimulating hormone receptor from B16 murine melanoma cells. *Biochem. J.* 286, 377–382.
- Anumula, K.R., Schulz, R. and Back, N. (1990) Immunologic methods for quantitative estimation of small peptides and their application to bradykinin. *J. Immunol. Methods* 135, 199–208.
- Avrameas, S. and Guilbert, B. (1972) Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates. *Biochimie* 54, 837–842.
- Ball, J.M., Henry, N.L., Montelaro, R.C. and Newman, M.J. (1994) A versatile synthetic peptide-based ELISA for identifying antibody epitopes. *J. Immunol. Methods* 171, 37–43.
- Belanger, L., Sylvestre, C. and Dufour, D. (1973) Enzyme-linked immunoassay for alpha-fetoprotein by competitive and sandwich procedures. *Clin. Chim. Acta* 48, 15–18.
- Berghorn, K.A., Bonnett, J.H. and Hoffman, G.E. (1994) cFos immunoreactivity is enhanced with biotin amplification. *J. Histochem. Cytochem.* 42, 12, 1635–42.
- Bleumink, E. and Young, E. (1968) Identification of the atopic allergen in cow's milk. *Int. Arch. Allergy* 34, 521–543.
- Boudet, F., Thèze, J. and Zouali, M. (1991) UV-treated polystyrene microtitre plates for use in an ELISA to measure antibodies against synthetic peptides. *J. Immunol. Methods* 142, 73–82.
- Bull, H. (1956) Adsorption of bovine serum albumin on glass. *Biochem. Biophys. Acta* 19, 464–71.
- Butler, J.E., McGivern, P.L. and Swanson, P. (1978) Amplification of the enzyme-linked immunosorbent assay (ELISA) in the detection of class-specific antibodies. *J. Immunol. Methods* 20, 365–83.
- Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R. and Cantarero, L.A. (1992) The physical behavior of capture antibodies adsorbed on polystyrene. *J. Immunol. Methods* 150, 77–90.
- Catt, K. and Tregear, G.W. (1967) Solid-phase radioimmunoassay in antibody-coated tubes. *Science* 158, 1570–1572.
- Cesbron-Delauw, Boutillon, C., Mercier, C., Fourmaux, M.P., Murray, A., Miquey, F., Tartar, A. and Capron, A. (1992) Amino acid sequence requirements for the epitope recognized by a monoclonal antibody reacting with the secreted antigen GP28.5 of *Toxoplasma gondii*. *Mol. Immunol.* 29, 1375–1382.
- Chang, Y., Wu, C., Chang, R. and Shinan, D. (1994) Determination of biotin concentration by a competitive enzyme-linked immunosorbent assay (ELISA) method. *J. Biochem. Biophys. Methods* 29, 321–29.
- Coursaget, P., Depril, N., Buisson, Y., Molinier, C. and Roue (1994) Hepatitis type E in a French population: detection of anti-HEV by a synthetic peptide-based enzyme-linked immunosorbent assay. *Res. Virol.* 145, 51–57.
- Diamandis, E.P., Christopoulos, T.K. (1991) The biotin-

- (strept)avidin system: principles and applications in biotechnology. *Clin. Chem.* 37, 625–636.
- Dierks, S.E., Butler, J.E. and Richerson, H.B. (1986) Altered recognition of surface-adsorbed compared to antigen-bound antibodies in the ELISA. *Mol. Immunol.* 23, 403–411.
- Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.
- Engvall E. (1980) Enzyme immunoassay: ELISA and EMIT. *Immunoassays* 419–439.
- Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunoassay, ELISA. Quantitative assay of immunoglobulin G. *Immunochemistry* 8, 871–874.
- Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunoassay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* 109, 129–135.
- Engvall, E., Jonsson, K. and Perlmann, P. (1971) Enzyme linked immunoassay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. *Biochim. Biophys. Acta* 251, 427–434.
- Fisher, P.M. and Howden, M.E.H. (1990) Direct enzyme-linked immunosorbent assay of anti-peptide antibodies using capture of biotinylated peptides by immobilized avidin. *J. Immunoassay* 11, 311–327.
- Frobert, Y and Grassi, J. (1992) Screening of monoclonal antibodies using antigens labelled with acetylcholinesterase. In: M. Manson (Ed.), *Methods in Molecular Biology*, Vol. 10, *Immunochemical Protocols*, Humana Press, Clifton, NJ, pp. 65–78.
- Gaudriault, G. and Vincent, J.P. (1992) Selective labeling of α - or ϵ -amino groups in peptides by the Bolton-Hunter reagent. *Peptides* 13, 1187–1192.
- Geerligs, H.J., Weijer, W.J., Bloemhoff, W., Welling, G.W. and Welling-Wester, S. (1988) The influence of pH and ionic strength on the coating of peptides of herpes simplex virus type 1 in an enzyme-linked immunosorbent assay. *J. Immunol. Methods* 106, 239–244.
- Goldman, A.S., Anderson, D.W., Sellers, W.A., Saperstein, S., Kniker, W.T. and Halpern, S.T. (1963) Milk allergy. I. Oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics* 32, 425–443.
- Grassi, J. (1994a) Interférences dues aux immunoglobulines, un poison pour tous les dosages immunologiques. I. Origine des anticorps anti-immunoglobulines et mécanisme des interférences. *Immunol. Biol. Spéc.* 9, 60–67.
- Grassi, J. (1994b) Interférences dues aux anticorps anti-immunoglobulines, un poison pour tous les dosages immunologiques. II. Interférences, mise en évidence et suppression. *Immunol. Biol. Spéc.* 9, 124–33.
- Grassi, J., Didierlaurent, A. and Stadler, B.M. (1986) Quantitative determination of total and specific human IgE with the use of monoclonal antibodies. *J. Allergy Clin. Immunol.* 77, 808–822.
- Grassi, J., Frobert, Y., Pradelles, P., Chercuitte, F., Gruaz, D., Dayer, J.M. and Poubelle, P.E. (1989) Production of monoclonal antibodies against interleukin-1 α and -1 β . Development of two enzyme immunometric assays (EIA) using acetylcholinesterase and their application to biological media. *J. Immunol. Methods* 123, 193–210.
- Grassi, J., Nevers, M.C., Frobert, Y., Poubelle, P. and Pradelles, P. (1990) Interférences observées dans des dosages immunologiques en présence d'anticorps anti-immunoglobulines. *Comptes rendus du VII^{ème} Colloque sur les Actualités en Immunoanalyse*, pp. 169–187, 17–20.
- Gruen, L.C., McKimm-Berschkin, J.L., Caldwell, J.B. and Nice, E.C. (1994) Affinity ranking of influenza neuraminidase mutants with monoclonal antibodies using an optical biosensor comparison with ELISA and slot blot assays. *J. Immunol. Methods* 168, 91–100.
- Guesdon, J.L., Ternynck, T. and Avrameas, S. (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* 27, 1131–1139.
- Hashida, S., Tanaka, K., Yamamoto, N., Uno, T., Yamaguchi, K. and Ishikawa, E. (1991) Detection of attomoles of [Arg8]-Vasopressin by novel noncompetitive enzyme immunoassay (hetero-two-site complex transfer enzyme immunoassay). *J. Biochem.* 110, 486–492.
- Hoffman, D.R. (1973) Estimation of serum IgE by an enzyme-linked immunosorbent assay (ELISA). *J. Allergy Clin. Immunol.* 51, 303–307.
- Ishikawa, E., Tanaka, K. and Hashida, S. (1990) Novel and sensitive noncompetitive (two-site) immunoassay for haptens with emphasis on peptides. *Clin. Biochem.* 22, 445–453.
- Ivanov, V.S., Suvorova, Z.K., Tchikin, L.D., Kozhich, A.T. and Ivanov, V.T. (1992) Effective method for synthetic peptide immobilization that increases the sensitivity and specificity of ELISA procedures. *J. Immunol. Methods* 153, 229–233.
- Metreau, E., Pleau, J.M., Dardenne, M., Bach, J.F. and Pradelles, P. (1987) An enzyme immunoassay for synthetic thymulin. *J. Immunol. Methods* 102, 233–242.
- Olivieri, V., Beccarini, L., Gallucci, G., Romano, T. and Santoro, F. (1993) Capture assay for specific IgE. An improved quantitative method. *J. Immunol. Methods* 157, 65–72.
- Plebani, A., Ugazio, A.G., Avanzini, A.M., Monafò, V. and Burgio, G.R. (1986) An enzyme-linked immunosorbent assay for cow's milk protein-specific IgE using biotinylated antigen. *J. Immunol. Methods* 90, 241–246.
- Pradelles, P., Grassi, J. and Maclof, J. (1985) Enzyme immunoassay of eicosanoids using acetylcholinesterase as label: an alternative to radioimmunoassay. *Anal. Chem.* 77, 1170–1173.
- Roy, A., Sharma, V.P. and Chauhan, V.S. (1994) The use of peptide ELISA in determining malaria endemicity. *J. Immunol. Methods* 167, 139–143.
- Schvartz, I., Gitlin, G., Amarant, T., Ittoop, O. and Hazum, E. (1991) Biotinylated endothelin as a probe for the endothelin receptor. *Peptides* 12, 1229–1233.
- Suter, M. and Butler, J.E. (1986) The immunochemistry of sandwich ELISAs. II. a novel system prevents the denaturation of capture antibodies. *Immunol. Lett.* 13, 313–316.
- Suter, M., Butler, J.E. and Peterman, J.H. (1989) The immunochemistry of sandwich ELISAs. III. The stoichiometry and efficacy of the protein-avidin-biotin capture (PABC) system. *Mol. Immunol.* 26, 221.

- Voller, A. (1975) Microplate enzyme-linked immunosorbent assay for Chagas' disease. *Lancet* February 22, 426–428.
- Wal, J.M., Bernard, H., Yvon, M., Peltre, G., David, B., Creminon, C., Frobert, Y. and Grassi, J. (1995) Enzyme immunoassay of specific human IgE to purified cows' milk allergens. *Food Agric. Immunol.* 7, 175–187.
- Wilchek, M. and Bayer, E.A (1990) Introduction to avidin-biotin technology. *Methods Enzymol.* 184, 5–51.
- Yorde, D.E., Sasse, E.A., Wang, T.Y., Hussa, R.O. and Garancis, J.C. (1976) Competitive enzyme-linked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. *Clin. Chem.* 22, 1372–1377.