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Measurement of the Reactive and Available Solid-Supported Carboxylic, *N*-Hydroxysuccinylated Carboxylic, and Aldehydo Groups

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ABSTRACT

We describe new colorimetric methods for the determination of the reactive and available solid-supported carboxylic, *N*-hydroxysuccinylated carboxylic, and aldehydo groups under conditions usually applied for the coupling of biomolecules. The methods involve the reaction of the solid-supported functional groups with tyramine or cysteine, and the subsequent titration of the ligand coupled onto the solid supports using the commercially available bicinchoninic acid/copper protein assay reagent (BCA). The titration is based on the ability of these ligands to reduce Cu^{2+} to Cu^{+}, which forms a chelate complex with bicinchoninic acid absorbing at 562 nm. The quantitation is finally carried out through standard curves obtained using tyramine or cysteine solutions of known concentrations. The values obtained by the assays developed for several solid supports carrying carboxylic, NHS-ester, and aldehydo groups were well correlated with those obtained by other literature methods or provided by the manufacturers. All of the proposed methods are simple, more sensitive than other relevant literature methods, and require only commericially available reagents.

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Index Entries: Colorimetric determination, of reactive solid-supported carboxylic, *N*-hydroxysuccinylated carboxylic, aldehydo groups; bicinchoninic acid, protein assay reagent, solid-supported carboxylic, *N*-hydroxysuccinylated carboxylic, aldehydo groups; solid supports, carrying carboxylic, *N*-hydroxysuccinylated carboxylic, aldehydo groups.

INTRODUCTION

Carboxylic groups, providing stable coupling of biomolecules through their amino groups, are among the most commonly used solid-supported functional groups (*1*). In addition, the solid-supported *N*-hydroxysuccinylated esters of carboxylic groups (NHS-esters) have become very popular since their use alleviates the need for activating reagents, such as carbodiimides, overcoming undesired crosslinking of ligands (*2*). On the other hand, solid supports carrying aldehydo groups have found wide applications as matrices for enzyme immobilization (*3*) and for affinity chromatographic techniques (*4*), since they provide low nonspecific binding, minimal leakage, and in some cases, stabilization of the insolubilized biomolecules (*5,6*).

The determination of the reactive and available solid supported functional groups is of great importance for optimizing the coupling conditions of ligands and for selecting the appropriate solid support for each specific application. Thus, several methods have been described in the literature for the determination of the functional groups mentioned above (*7–10*). However, depending on the method, certain disadvantages are associated with them, such as low sensitivity, tedious protocols, use of radiolabeled compounds, and/or requirement of special instrumentation (e.g, amino acid analyzer).

Recently, we reported simple and sensitive one-step methods (*11*) for the colorimetric determination of the total solid-supported aldehydo and NHS-ester groups based on their ability to produce color directiy when incubated with the bicinchoninic acid (BCA)/copper protein assay reagent (*12*). During the development of those methods, we found that this reagent offered several advantages over other colorigenic reagents used in relevant protocols, such as high sensitivity of the assays, stability of the stock reagents for more than 6 mo, and stability of the chromophore produced for more than 3 h, in addition to producing color when incubated with a variety of substances. Although these methods have several advantages compared with others proposed in the literature, it is well known that only a fraction of the total functional groups is available for coupling with ligands under certain reaction conditions. Thus, regarding the user's point of view, the determination of the reactive and available solid-supported functional groups is considered of great importance. For this reason, given the above-mentioned advantages offered by the BCA

reagent, we developed simple and sensitive methods for the determination of the reactive and available solid-supported carboxylic, NHS-ester, and aldehydo groups based on the reaction of these groups with appropriate ligands that can be titrated using this reagent.

MATERIALS AND METHODS

Materials

4,4′-Dicarboxy-2,2′-biquinoline sodium salt (bicinchoninic acid, BCA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), tyramine-HCl, cysteine-HCl, cystamine-HCl, $NaBH_4$, along with ϵ-amino-*n*-caproic acid-agarose and NHS-chloroformate-activated agarose gels were obtained from Sigma Chemical Co. (St. Louis, MO). AminoLink gel was purchased from Pierce Chemical Co. (Rockford, IL). CH Sepharose 4B, Activated CH Sepharose 4B, CM Sepharose CL-6B, and Sepharose 4B gels were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). Affi-Gel 10 and CM Bio-Gel A gels were from Bio-Rad (Richmond, CA). Ethylenediamine, $NaBH_3CN$, and all the other reagents and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany), unless otherwise indicated. The water used throughout this study was doubly distilled.

Reagents

BCA assay reagents: A, 1% BCA, 2% $Na_2CO_3 \cdot H_2O$, 0.16% sodium tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$, pH, 11.25, and B, 4% $CuSO_4 \cdot 5H_2O$, were prepared according to Smith et al. (*12*), and were filtered through 0.45-μm filters (Millipore). The BCA working solution was obtained by mixing 50 parts of reagent A with 1 part of reagent B immediately before use.

Methods

Pretreatment of the Solid Supports

The solid supports (100 mg dry or 500 μL of 50% slurry) containing carboxylic or aldehydo groups were washed with 2 × 10 mL of 0.5*M* NaCl and 2 × 10 mL of distilled water, whereas the gels containing NHS-esters were washed with 4 × 10 mL of cold 0.001*M* HCl just before use.

Preparation of Solid-Supported Aldehydo Groups

Two-milliliter portions of packed washed Sepharose 4B gel were suspended in flasks containing 40 mL of distilled water. Appropriate amounts of 0.1*M* $NaIO_4$ solution in water were added to provide final concentrations of 0.5, 2.5, and 10 m*M*, and the suspensions were gently stirred for 1 h at 25°C. Then, the supernates were removed, and 5 mL of 50% (v/v)

propyleneglycol were added to the solids to consume the remaining unreacted $NaIO_4$. After that the gels were filtered and extensively washed with distilled water. The activated aldehydo-Sepharose 4B gels prepared, which will thereafter be called ALI, ALII, and ALIII (corresponding to the gels activated with the above-described concentrations of $NaIO_4$), were immediately used for coupling of ligands.

Determination of the Solid-Supported Carboxylic Groups

The coupling of tyramine onto solid supports carrying carboxylic groups was carried out by adding 1 mL of 0.1*M* tyramine solution, pH 4.0, to a tube containing 100 μL of the packed washed gel carrying carboxylic groups, and the tube was tumbled end-over-end for 5 min. Then 500 μmol of solid EDC were added to the suspension and stirred for 30 min while the pH was monitored and maintained at 4–5 by adding HCI. After that, another 500-μmol portion of EDC was added, and the pH was again maintained at 4–5 for 15 min. Then, the gel was tumbled for 15 more min at room temperature and washed successively with 2 × 10 mL of 0.1*M* sodium phosphate buffer, pH 7.0, and 3 × 10 mL of 0.25*M* sodium carbonate/bicarbonate buffer, pH 11.25, prior to the determination of the tyramine coupled to the solid using the BCA assay.

Determination of the Solid-Supported N-Hydroxysuccinimidyl Carboxylates

One hundred microliters of packed washed gel carrying NHS-ester groups were incubated with 1 mL of 0.1*M* tyramine solution of 0.01*M* sodium carbonate buffer, pH 8.5, for 1 h under continuous shaking. Then, the supernate was aspirated, and the gel was washed successively with 2 × 10 mL of 0.1 *M* sodium phospate buffer, pH 7.0, 1 × 4 mL of 0.1*M* NH_4OH solution (10-min incubation with shaking), and finally with 3 × 10 mL of 0.25*M* sodium carbonate/bicarbonate buffer, pH 11.25. Then, the tyramine coupled onto the solids was estimated using the BCA assay.

A similar protocol was followed when the reaction was carried out in anhydrous methanol, but the pH of tyramine solution was adjusted with methanolic NaOH, and the first washing solution was replaced by methanol. All the other steps of the protocol were as described above.

Determination of the Reactive and Available Solid-Supported Aldehydo Groups

One milliliter of 200 m*M* tyramine or cysteine solution in 0.1*M* sodium phosphate buffer (final pH 6.5) and 50 μL of 1*M* $NaBH_3CN$ solution in water were added to tubes containing 100 μL of the packed washed gels carrying aldehydo groups, and they were incubated overnight at 22°C under mild shaking. Then, after adjusting the pH to 10.0 by adding Na_2CO_3, 20 mg of solid $NaBH_4$ were added to the tubes, and they were incubated for 2 more hours under shaking. After that, the solids were washed with 3 × 10 mL of 0.1*M* sodium phosphate buffer, pH 6.5, and

3 × 10 mL of 0.25*M* sodium carbonate buffer, pH 11.25, and then the tyramine or cysteine coupled onto the solids was determined by the BCA assay.

BCA Assay (Detection Protocol)

After the reaction with tyramine or cysteine, the packed washed gel (100 μL) was diluted to a final volume of 1 mL with 0.25*M* sodium carbonate/bicarbonate buffer, pH 11.25. After vortexing, 100 μL of the suspension (corresponding to 10 μL of gel) were transferred to plastic tubes, and 2 mL of BCA working solution were added into each tube. The tubes were vortexed and incubated with continuous shaking for 1 h at 60°C. Then, the tubes were centrifuged and, after cooling to room temperature, the absorbance of the supernates was measured at 562 nm against a reagent blank.

Control Samples

The absorbance provided by a 4% beaded agarose (Sepharose 4B), not containing functional groups, was used as blank value. The effectiveness of the washing protocols to eliminate the nonspecific binding (NSB) and possible interference of the reagents in the color formation was investigated using Sepharose 4B treated exactly as each one of the functionalized supports (control sample).

Standard Curves

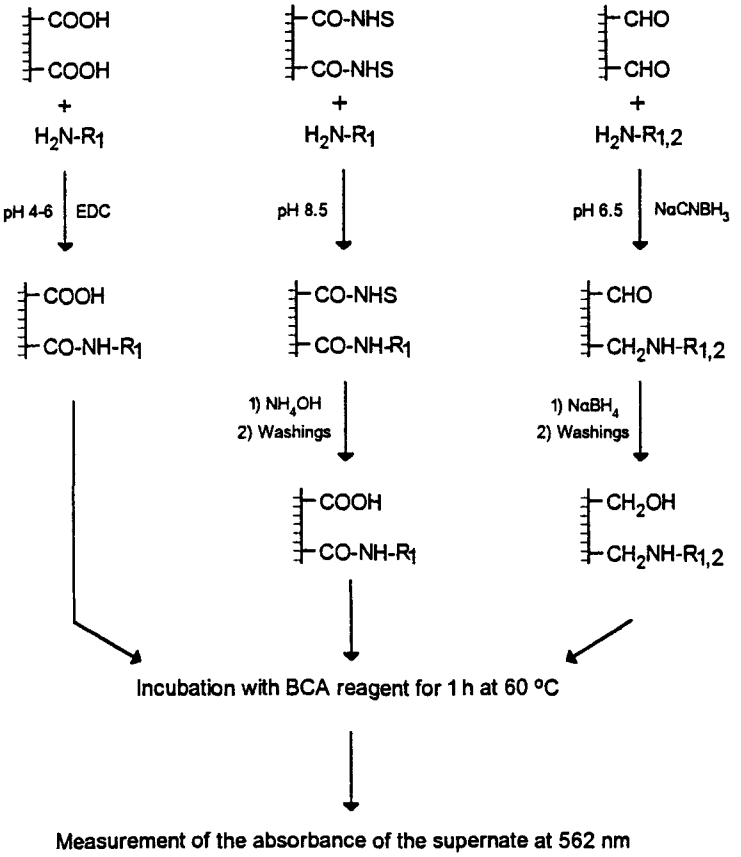
Standard solutions of tyramine or cysteine prepared in 0.25*M* sodium carbonate/bicarbonate buffer, pH 11.25, were used for the quantitation of the ligands coupled onto the solid surfaces. For both ligands, the concentrations of the standard solutions ranged between 0.02 and 1.5 m*M*. Standard curves were made up by incubating in triplicate 100 μL of the standard solutions with 2 mL of BCA working solution for 1 h at 60°C and determining the absorbance of the solutions at 562 nm.

Other Literature Methods

Comparative determination of the reactive and available solid-supported carboxylic groups was carried out following the method of Ngo (*8*), but the reaction with cystamine proceeded for only 1 h instead of 24 h. The reactive aldehydo groups content of the solid supports was also estimated following the protocol described by Guisan (*10*). The determination of the total solid-supported NHS-ester and aldehydo groups was performed by a recently reported protocol (*11*) using BCA reagent.

RESULTS AND DISCUSSION

In this article, we describe new colorimetric methods for determination of the reactive and available solid-supported carboxylic, NHS-ester, and aldehydo groups. According to the methods (Scheme 1), appropriate



Scheme 1. Flowchart of the protocols proposed for the determination of the reactive and available solid-suppported carboxylic, NHS-ester, and aldehydo groups (H_2N-R_1:tryamine, H_2N-$R_{1,2}$:tyramine or cysteine).

ligands (e.g., tyramine or cysteine) react with the solid-supported functional groups and introduce onto the solid surfaces groups possessing the ability to reduce Cu^{2+} to Cu^{+}. Then, the uncoupled reagents are removed, and the unreacted solid-supported functional groups are transformed (if required) to groups that do not interfere in the measurement. After that, the solids are incubated with BCA/$CuSO_4$ protein assay reagent, and the Cu^{+}, which is produced in the solution by the coupled substance, forms a chelate complex with the BCA absorbing at 562 nm. The quantitation of the groups introduced on the solid surface is finally carried out through the standard curve of the corresponding substance, which is used for coupling (Fig. 1).

Selection of the Ligands

The developed assays require the use of appropriate ligands that can react with the solid-supported functional groups and provide color when incubated with the BCA reagent. For this purpose, we tested several sub-

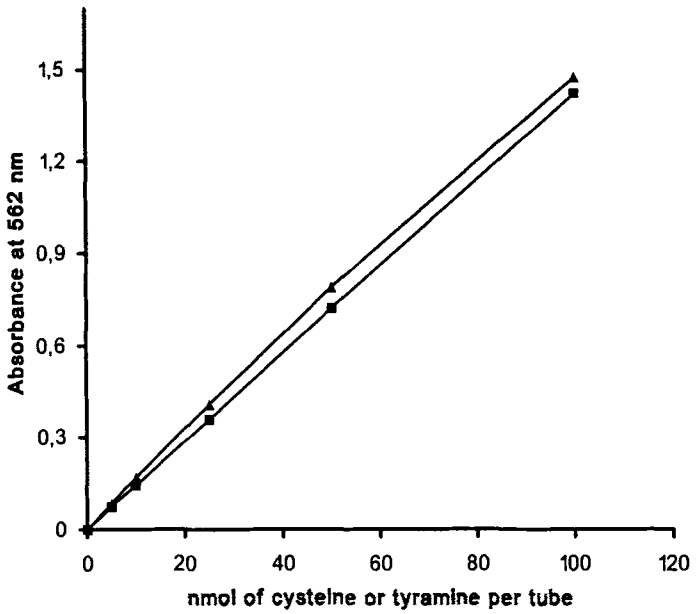


Fig. 1. Tyramine (▲) and cysteine (■) standard curves obtained by the BCA assay.

stances that meet these requirements, and finally, we selected tyramine and cysteine since they are commercially available and provide more intense color with the BCA reagent than other substances, resulting in assays of high sensitivity. Our preliminary experiments indicated that tyramine can be coupled equally well with all kinds of the functional groups used in this study. On the other hand, cysteine was appropriate for coupling with the aldehydo groups, but provided inconsistent and unreliable results when used as ligand for the determination of carboxylic groups, mainly because of the crosslinking of cysteine molecules. Additionally, concerning the NHS-ester groups we found that although it was feasible to determine the amount of cysteine coupled onto the solids, the precision of the determination was poor. This was possibly because of the coupling of cysteine through its sulfhydryl group (*9*), which, however, should be uncoupled in order to produce color with the BCA reagent. For all these reasons, in our protocols, we adopted tyramine as ligand for the determination of the reactive and available solid-supported carboxylic and NHS-ester groups, whereas both tyramine and cysteine were used for the determination of the reactive and available aldehydo groups.

Optimization of the Conditions of the Assays

We tested several parameters that could affect the performance of the assays, such as the pH and the duration of the coupling reactions, the concentrations of the ligands, the washing/transforming procedure, and the kinetics of the color formation.

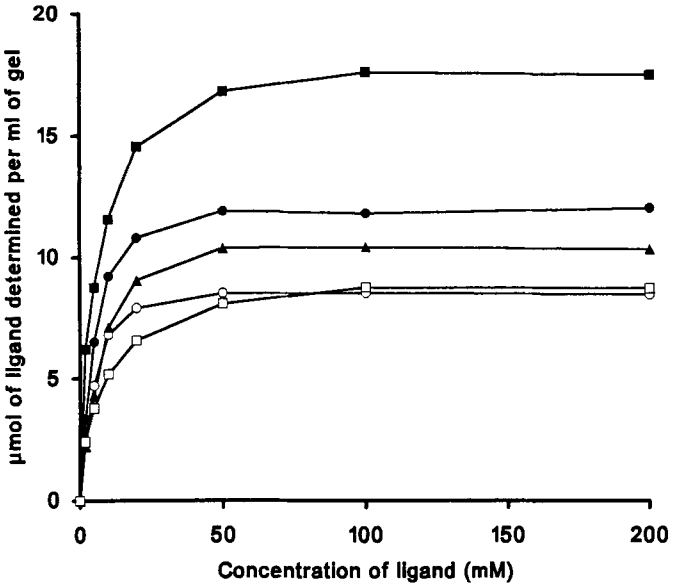


Fig. 2. Reactive and available groups determined using increasing concentrations of tyramine for CH Sepharose (▲) and for Activated CH Sepharose (under aqueous, ○, or anhydrous, ●, conditions), and using increasing concentrations of tyramine (□) and cysteine (■) for AminoLink gel.

We found that by maintaining the pH of the reaction of tyramine with carboxylic groups between 4 and 5, maximum coupling values were obtained. On the other hand, using the NHS-ester groups, maximum coupling was obtained at pH 8.2–8.7, whereas the solid-supported aldehydo groups provided maximum plateau coupling values when the pH of the reaction ranged between 6.2 and 7.4. All these findings are in good agreement with the results of previously published works (*8,9,13,14*), and thus, in our protocols we adopted the pHs that are most widely used in the literature for coupling of biomolecules.

Using gels containing carboxylic groups (e.g., CH Sepharose, CM Sepharose), we found that 1 h of reaction with tyramine was required to achieve maximum coupling values, whereas a 20-min reaction was adequate for the solid-supported NHS-esters. Very similar findings, with respect to the reaction time, were obtained using cystamine instead of tyramine for the comparison method used for the determination of the reactive and available carboxylic groups (*8*). On the other hand, using supports carrying aldehydo groups, maximum coupling values were obtained after 18 h of reaction with either tyramine or cysteine.

Following the conditions described under Materials and Methods for each kind of the supports, we found that maximum coupling was obtained using tyramine concentrations equal to or higher than 50 m*M* for both the solid-supported carboxylic and NHS-ester groups, whereas 100-m*M* or higher concentrations of tyramine or cysteine were required to achieve maximum coupling values for the aldehydo-containing supports (Fig. 2).

Especially for the NHS-ester groups, we found that when the reaction of tyramine was performed in anhydrous environment, the values obtained were almost 25% higher compared with those obtained when the reaction was carried out in aqueous milieu, mainly because of the hydrolysis of the NHS-esters in the latter medium.

For reliable results, when the BCA is used as detection reagent for the determination of the reactive and available solid-supported functional groups, both the solid support and the unreacted functional groups remaining on the solid surface should not contribute by themselves to the color formation. The solid matrices used in this study were agarose-based materials, which do not provide color with the BCA reagent as indicated by the absorption values (< 0.015) provided by Sepharose 4B (blank value). The same results were obtained when using all of the supports carrying carboxylic groups. On the contrary, aldehydo and NHS-ester groups provide color when incubated with this reagent (*11*). In case of aldehydo groups, we overcame this problem by introducing in our protocol a reduction step employing $NaBH_4$, after the coupling reaction, in order to convert the aldehydo groups to hydroxylic groups, which do not produce color in the BCA assay. We found that this step eliminated the remaining aldehydo groups, since the absorbance values provided by the reduced materials (≤ 0.015) were the same as those provided by Sepharose 4B (blank value). The same results were obtained using the solids carrying NHS-ester groups after their treatment for 5 min with NH_4OH solution, and thus, such a step was included in the washing protocol proposed for these supports in order to remove any of the NHS-ester groups remaining on the solid surface after the reaction with tyramine.

A possible drawback encountered in several methods used for the determination of the reactive and available functional groups is the nonspecific binding of the ligand and/or other reagents involved in the protocol, since their presence during the detection step can significantly influence the results. We tested several washing procedures in order to decrease the nonspecific binding of tyramine, cysteine and of other reducing agents (e.g., $NaBH_4$, $NaBH_3CN$) used in our protocols. We found that following the procedures described under Materials and Methods, the absorbance value (< 0.020) provided by the Sepharose 4B when treated exactly as the samples carrying functional groups (following the specific procedure adopted for each kind of functional group) was very low (< 0.020). This indicated that all of the proposed washing protocols were capable of completely removing the ligands and/or the reagents nonspecifically bound onto the solids. However, in order to avoid any false overestimation, we subtracted this value from those obtained by the test samples.

Concerning the detection step, we studied the kinetics of the color formation for both the standards and the solid supports. We found that 30 min of incubation with BCA reagent at 60°C was adequate for either tyramine or cysteine standards to reach plateau absorbance values, whereas in all cases, the immobilized substances required 1 h of incubation. Addi-

tionally, the color, once formed, remained unaffected for several hours. Thus, a 1-h incubation at 60°C was selected for both the standards and the solid supports.

Characteristics of the Assays

The standard curves provided by the assays were very consistent for a long period of time when the same BCA stock solutions were used (data not shown). Thus, since the BCA stock solutions are stable for more than 6 mo (*11,15*), it is not considered necessary to run a standard curve every time together with the unknown samples, although, a control sample should be included in the test to ascertain possible reagent failure.

The assays developed were characterized by high sensitivity and a wide dynamic range, providing the ability to determine amounts of tyramine or cysteine ranged between 1 and 150 nmol/assay tube without dilution of the final solution. The high sensitivity of the assays is in apparent contradiction with the relatively low extinction coefficient of Cu^+-BCA complex, which is $6590M^{-1}\cdot cm^{-1}$ (*16*); however, by calculating the slopes of the tyramine and cysteine standard curves ($32{,}760M^{-1}\cdot cm^{-1}$ and $30{,}035M^{-1}\cdot cm^{-1}$, respectively), we found that they were four to fivefold higher than the extinction coefficient of the Cu^+-BCA complex. Although we do not have a solid explanation for this phenomenon, it seems that during incubation of tyramine or cysteine (at this range of concentrations) with the BCA reagent at 60°C, approx 4–5 Cu^+ ions are produced in the solution/molecule of ligand, resulting in high sensitivity of the assays developed.

We evaluated the linearity of each assay by determining the solid-supported functional groups or ligands using increasing volumes of the corresponding gels. The linear regression equations obtained for CH Sepharose, Activated CH Sepharose, and AminoLink gels by plotting the measured tyramine valves (y axis) against those expected, considering the value determined using the higher volume of the gel (x axis), were $y = 1.04x - 0.07$ (R: 0.997, $p < 0.001$), $y = 0.99x + 0.05$ (R: 0.996, $p < 0.001$), and $y = 1.01x + 0.06$ (R: 0.997, $p < 0.001$), respectively. Likewise, excellent linearity was also obtained with all the other solid supports tested.

Measurement of the Reactive and Available Solid-Supported Carboxylic, NHS-Ester, and Aldehydo Groups

Using the methods developed, we determined the coupling capacity of several solid supports carrying carboxylic, NHS-ester, and aldehydo groups. As shown in Table 1, the values determined by the method developed for the solid-supported carboxylic groups were well correlated with those provided by the comparison method and lay within the ranges given by the manufacturers. Nevertheless, our method provides twofold higher sensitivity and seems to be more convenient than the comparison one, since it does not suffer from other problems associated with the

Table 1
Colorimetric Determination of Solid-Supported Carboxylic and NHS-Ester Groups (mmol/mL gel)

Solid supports	Functional group	Reactive and available groups		Total NHS-ester groups	Manufacturers' values[a]
		BCA assay	Comparison method		
Sepharose 4B	—	0.0	0.0	0.0	—
CH Sepharose 4B	COOH	10.4 ± 0.3[b]	9.9 ± 0.2	—	10–14
e-Amino-*n*-caproic acid-agarose	COOH	8.5 ± 0.2	8.1 ± 0.2	—	—
CM Sepharose CL-6B	COOH	109.7 ± 3.2	115.2 ± 2.8	—	100–140
CM Bio-Gel A	COOH	17.6 ± 3.2	18.2 ± 0.5	—	approx 20
Affi-Gel 10	NHS-ester	9.8 ± 0.2 (13.7 ± 0.3)[c]	—	16.3 ± 0.6	16–18
Activated CH Sepharose 4B	NHS-ester	8.5 ± 0.3 (12.1 ± 0.3)	—	15.2 ± 0.4	9–15
NHS-chloroformate-activated agarose	NHS-ester	4.8 ± 0.2 (7.1 ± 0.2)	—	10.1 ± 0.4	—

[a] Where available.
[b] Mean value of eight independent determinations (±SD, $p < 0.01$). Three replicates were used for each determination.
[c] Values in parentheses obtained when the coupling was carried out in anhydrous methanol.

colorigenic reagent used in this method (DTNB), e.g., inaccuracy owing to autohydrolysis and/or thermochromism, described elsewhere (*17,18*).

Considering the solid supports carrying NHS-ester groups, we found that the values obtained by the method developed when the coupling reaction was performed in aqueous medium were lower than the minimum value of the ranges given by the manufacturers. This was also true for Affi-Gel 10, even in the case that the reaction with tyramine took place in an anhydrous environment. However, as has been described previously (*11*) and can be also concluded by the results presented in Table 1, the values provided by the manufacturers for these supports refer to the total amount of the NHS-ester groups existing on the supports, and not to the reactive and available ones. Thus, the method developed provides helpful information to the users concerning the coupling of ligands onto these supports under certain reaction conditions and is more convenient than the literature method (*9*), which uses a radioactive substance as the ligand. Additionally, the method gave us the ability to gain insight concerning the availability of the functional groups for reaction with low-mol-wt ligands. We found that using aqueous coupling reaction conditions, the coupling capacity values obtained for the NHS-chloroformate-activated agarose, the Activated CH Sepharose 4B, and the Affi-Gel 10 corresponded to 48, 56, and 60% of the total NHS-ester groups content of these supports, respectively. A similar trend was also observed when anhydrous coupling conditions were used (corresponding values: 70, 80, 84%). Since the NHS-ester groups of those solid supports are joined with the polymeric backbone through 1-, 6-, and 10-atoms spacers, respectively, this trend can be ascribed, at least to some extent, to the differences in the spacer arms' length.

In Table 2 are presented the values of the reactive and available aldehydo groups obtained by the methods developed using tyramine or cysteine as ligands and the values obtained by a method described in the literature using ethylenediamine for coupling and TNBS for the detection step (*10*). The total aldehydo group values of those supports are also provided. As is shown, the values provided for all the solid supports tested using tyramine as ligand were in good agreement with those obtained by the comparison method, whereas significantly higher values were obtained using cysteine. Since there are no significant differences between cysteine and tyramine concerning their molecular weights, the differences between their values are, to some extent, unexpected. However, these supports are most often prepared by direct oxidation of polysaccharic gels yielding aldehydo groups anchored either directly or through one C-atom spacer to the polysaccharic backbone (*5,8*). Since we found that the values of the reactive and available groups determined using the methods developed as well as the comparison method corresponded in all cases to $< 18\%$ of the total aldehydo group values estimated for these supports, it seems that the essential lack of spacer arm results in low availability of the aldehydo groups for reaction. Thus, the differences observed between

Table 2
Colorimetric Determination of Solid-Supported Aldehydo Groups

Solid support	Reactive and available aldehydo groups, mmol/mL gel			Total aldehydo groups, mmol/mL gel
	Tyramine/BCA	Cysteine/BCA	Comparison method[a]	
Sepharose 4B	0.00	0.00	0.00	0.00
AminoLink	8.6 ± 0.2 [b]	17.5 ± 0.4	8.7 ± 0.2	195 ± 8
ALI[c]	0.22 ± 0.01	0.43 ± 0.03	0.19 ± 0.01	2.5 ± 0.1
ALII	0.52 ± 0.03	0.85 ± 0.04	0.56 ± 0.04	8.1 ± 0.3
ALIII	0.90 ± 0.04	1.24 ± 0.05	0.92 ± 0.05	20.4 ± 0.4

[a]The comparative determination was carried out using ethylenediamine for coupling with the aldehydo groups and TNBS for the titration of the free amino groups introduced onto the solids (ref. *10*).

[b]Mean value of six independent determinations (± SD, $p < 0.01$). Three replicates were used for each determination.

[c]ALI, ALII, and ALIII represent aldehydo-Sepharose 4B gels prepared in-house by oxidation of Sepharose 4B using increasing concentrations of $NaIO_4$ (*see* Materials and Methods).

the values obtained using cysteine and tyramine as ligands can be also ascribed to steric hindrance effects. However, since these two substances have very similar molecular weights, our results indicate that the chemical structure of the ligand (e.g., the phenolic ring of tyramine and the analogous chemical structure of TNBS) could strongly influence its interaction with the solid-supported aldehydo groups.

Both of the methods developed provide at least twofold higher sensitivity compared with the literature method that uses TNBS for the determination of ethylenediamine coupled onto the aldehydo groups (extinction coefficient of TNBS 14,000$M^{-1}\cdot cm^{-1}$ at 340 nm) (*10*), and they are also devoid of harsh steps, such as the acidic hydrolysis of the gels at 120°C. For the determination of the reactive and available aldehydo groups, we propose the use of cysteine as ligand, since it provides significantly higher values than tyramine. Nevertheless, the combined use of these two ligands can provide more information concerning the coupling of low-mol-wt substances onto certain solid supports carrying aldehydo groups.

CONCLUSION

We developed methods for the determination of the reactive and available solid-supported carboxylic, NHS-ester, and aldehydo groups using tyramine or cysteine as ligand and the BCA protein assay reagent for their colorimetric determination. The methods are simple, accurate, and very sensitive allowing the determination of 1 nmol of reactive and available functional groups/solid support sample. Additionally, since the BCA stock solutions are commercially available and stable for more than 6 mo, the proposed methods should be easily adaptable for routine determinations and useful for the evaluation of solid supports.

REFERENCES

1. Inman, J. K. (1985), in *Affinity Chromatography: A Practical Approach*, Dean, P. D. G., Johnson, W. S., and Middle, F. A., eds., IRL, Oxford, pp. 53–59.
2. van Sommersen, A. P. G., Machielsen, P. A. G. M., and Gribnau, T. C. J. (1993), *J. Chromatogr.* **639,** 23–31.
3. Kennedy, J. F. (1985), *Handbook of Enzyme Biotechnology*, 2nd ed., Ellis Horwood, Chichester, pp. 147–207.
4. Margel, S. and Offarim, M. (1983), *Anal. Biochem.* **128,** 342–350.
5. Domen, P. L., Nevens, J. R., Mallia, A. K., Hermanson, G. T., and Klenk, D. C. (1990), *J. Chromatogr.* **510,** 293–302.
6. Guisan, J. M., Alvaro, G., Fernadez-Lafuente, R., Rossel, C. M., Garcia, J. L., and Tagliani, A. (1993), *Biotechnol. Bioeng.* **42,** 455–464.

7. Goldstein, L. (1973), *Biochim. Biophys. Acta.* **515,** 1–17.
8. Ngo, T. T. (1986), *Appl. Biochem. Biotechnol.* **13,** 207–212.
9. Cuatrecasas, P. and Parikh, I. (1972), *Biochemistry* **12,** 2291–2299.
10. Guisan, J. M. (1988), *Enzyme Microbiol. Technol.* **10,** 375–382.
11. Tyllianakis, P. E., Kakabakos, S. E., Evangelatos, G. P., and Ithakissios, D. S. (1994), *Anal. Biochem.* **219,** 335–340.
12. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985), *Anal. Biochem.* **150,** 76–85.
13. Carraway, K. L. and Triplett, R. B. (1970), *Biochim. Biophys. Acta* **200,** 564–566.
14. Borch, R. F., Bernstein, M. D., and Durst, H. D. (1971), *J. Am. Chem.Soc.* **93,** 2897–2904.
15. Kakabakos, S. E., Tyllianakis, P. E., Evangelatos, G. P., and Ithakissios, D. S. (1994), *Biomaterials* **15,** 289–297.
16. Hill, H. D. and Straka, J. G. (1988), *Anal. Biochem.* **170,** 203–208.
17. Tyllianakis, P. E., Kakabakos, S. E., Evangelatos, G. P., and Ithakissios, D. S. (1993), *Appl. Biochem. Biotech.* **34,** 15–25.
18. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979), *Anal. Biochem.* **94,** 75–81.