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Journal of Chromatography A, 868 (2000) 85–99

JOURNAL OF CHROMATOGRAPHY A

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Protein adsorption to the bare silica wall in capillary electrophoresis Quantitative study on the chemical composition of the background electrolyte for minimising the phenomenon

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Received 4 August 1999; received in revised form 26 October 1999; accepted 15 November 1999

Abstract

A novel method is reported for quantifying protein adsorption to naked silica tubings and for assessing the efficacy of amino quenchers added to the background electrolyte. It consists of flushing a fluorescently-labelled protein (myoglobin) into a capillary equilibrated in Tris–acetate buffer, pH 5.0, until full saturation of the potential adsorbing sites. Desorption is then affected by driving electrophoretically sodium dodecyl sulphate (SDS) micelles into the capillary from the cathodic reservoir: the peak of eluted material is quantified fluorometrically by using a dual laser beam instrument able to read the fluorescein-isothiocyanate-labelled myoglobin at 520 nm and the internal standard (sulphorodamine) at 630 nm. As potential quenchers, a series of monoamines have been investigated (triethylamine, triethanolamine, ethylamine), followed by diamines (putrescine, cadaverine and hexamethonium bromide) and finally by oligoamines [spermidine, spermine and TEPA (tetraethylenepentamine), i.e., a tri- a tetra- and a pentamine, respectively]. Two values of molarities have been derived: a value at 50% (a kind of a dissociation constant) and a value at 90% inhibition of binding of macromolecules to the silica surface. According to these figures of merit, mono- and diamines are rather poor quenchers of interaction with the wall, since the 50% values are of the order of 50–100 m*M* and the 90% values reach as high as 560 m*M*. On the contrary, oligoamines, especially spermine and TEPA, are most effective, since the 50% molarities are in the sub-millimolar range and the 90% values are of the order of ca. 1 m*M*. Figures of merit have also been derived for different washing procedures. Those most commonly adopted in routine practice, i.e., of washing with either 1 *M* NaOH or with 1 *M* HCl, or with both, leave behind traces of proteins still bound to the wall, whereas the SDS micelle electrophoretic desorption seems to be 100% effective.
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Keywords: Background electrolyte composition; Protein adsorption; Amines; Proteins

1. Introduction

Protein and peptide separations by capillary zone electrophoresis (CZE) have now become widely accepted by the scientific community at large, due to some inherent advantages of the method, such as very high resolution, fast analysis times, minimal sample requirement, negligible waste of toxic chemicals, high sensitivity on-line detection in the lower UV range (200 to 214 nm) [1,2]. The methodology has not been an easy one to implement, though, and

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PII: S0021-9673(99)01207-8

it has required continuous refinements over the years for attaining the present levels. The main obstacle to such separations was the tenacious affinity of ionised silanols for a proteinaceous surface: due to the multiple ionisation occurring onto the silica surface at any pH above 3 and to the multiple charges typically present on a protein surface, co-operative, multi-point attachment would ensue, resulting in a chromatographic adsorption process capable of seriously impairing the resolution. Aware of this problem, in 1967 Hjertén [3] proposed an inner surface coating capable of minimising such noxious interactions. It consisted of thermal immobilization of a neutral polymer, methylcellulose, followed by a cross-linking reaction with formaldehyde. Other, simpler strategies consist of the use of electrolyte solutions at extreme pH values, whether acidic, for suppressing silanol dissociation [4], or higher that the protein isoelectric point (p*I*), for conditioning both the proteins and the capillary wall to a negatively-charged state, thus provoking electrostatic repulsion [5]. In line with this strategy, our group has proposed, over the years, a number of amphoteric, isoelectric, acidic buffers, in an attempt to obtain silanol protonation while simultaneously reducing the high conductivity associated with conventional buffers (e.g., phosphate, pH 2.0 to 2.5) typically adopted for this purpose. The first one suggested was isoelectric aspartic acid, as the sole background electrolyte, operating at pH=p*I*=2.77 (at 25°C) [6], soon to be followed by iminodiacetic acid (IDA, pH=p*I*=2.23) [7] and by cysteic acid (pH=p*I*=1.85) [8]. The method was successfully used for a number of applications: for generating peptide maps of α- and β-globin chains from tryptic digests of human adult hemoglobin [9], for analysis of gliadins in the screening of wheat cultivars [10] and of zeins in maize [11,12] and for human globin chain separation and quantitation [13,14]. The fundamental properties of such buffers have been elaborated [15] and a number of reviews have already covered the field [16–18]. These were not the only strategies adopted: in an attempt at minimising protein adsorption without having to resort to chemical modification of the wall, via covalent attachment of various polymers, scientists have resorted to various strategies, mostly consisting of altering the composition of the background electrolyte, via a number of additives supposed to quench analyte adsorption. These ranged from zwitterions, to a series of amines (mono- to oligo-) to addition of neutral and charged polymers and surfactants. Since the literature in this field is quite large, we refer the readers to two reviews covering these aspects [19,20].

Among all the possible additives, perhaps the class which has received the greatest attention is that of the amine modifiers, ever since Nahum and Horváth [21] recommended them as additives to the mobile phases employed in reversed-phase chromatography of ionogenic substances, in order to suppress the untoward effect of the residual unmasked silanolic groups in the stationary phase. This class comprises a vast number of compounds, starting from monoamines, such as triethylamine and propylamine [22,23], morpholine [24], glucosamine and galactosamine [25], *N*,*N*-diethylethanolamine, *N*-ethyldiethanolamine, triethanolamine [26], ethanolamine [27], hydroxylamine, ethylamine [28] as well as the quaternary base tetramethylammonium chloride [29]. Among the diamines: 1,3-diaminopropane [30], 1,4-diaminobutane (putrescine) [31], 1,5-diaminopentane (cadaverine) [32], ethylendiamine [33], *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine [34] and the α,ω-bis-quaternary ammonium alkanes, such as hexamethonium [35] and decamethonium bromides [36]. In the family of oligoamines, we can recall: diethylentriamine and triethylentetramine [37], *N*,*N*′-bis(3-aminopropyl)1,4-butanediamine (spermine) [28] and 1,4,7,10-tetraazocyclodecane (cyclen) [38]. Finally, among the polyamines, we can list: chitosan [39], polyethylenimine [40] and polydimethylallyl ammonium chloride [41].

2. Materials and methods

2.1. Chemicals

Ethylamine, triethylamine, triethanolamine, spermine, spermidine, glucosamine, galactosamine and horse skeletal muscle myoglobin were from Sigma, St. Louis, MO, USA. Fluorescein isothiocyanate (FITC), dimethylformamide (DMF), hydroxylamine, sodium dodecyl sulphate (SDS), putrescine, cadaverine and hexamethonium bromide (HMB) were from Fluka, Buchs, Switzerland. Fused-silica capillaries

(100 μm I.D.×375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Protein labelling

Labelling of myoglobin with FITC has been performed according to the protocol in Ref. [42]. Briefly, myoglobin (5 mg/ml) is dissolved in 0.1 *M* sodium hydrogencarbonate, pH 9.0. FITC (10 mg/ml) is dissolved in DMF under vigorous stirring. Gently add 100 μl of FITC to 1 ml of protein solution, under constant stirring. Incubate for 1 h at room temperature. The reaction is blocked by adding 1 ml of 1.5 *M* hydroxylamine–HCl, pH 8.5, per ml of reaction mixture. The derivatized protein is then purified by gel filtration on a Sephadex G-25 column, equilibrated with PBS (phosphate-buffered saline, pH 7.4). The FITC-derivatized protein can be dispensed in aliquots and stored frozen at −70°C for at least 6 months.

2.3. Electrophoretic protein desorption

In order to measure the amount of protein bound to the capillary wall at any pH value of the background electrolyte, we exploited the method of Barberi et al. [43], consisting of desorption of bound material via sweeping the silica surface with SDS micelles, driven electrophoretically into the lumen from the cathodic reservoir. For the experiments given here, a final pH of 5.0 was chosen for the background electrolyte, since at this pH myoglobin is well below the p*I* (=7.6), so it has a substantial positive charge, whereas the silica is strongly negatively charged, since this pH value closely matches the average p*K* of silanols, given by Bello et al. as 5.3 [44]. The experiments proceed as follows: a 4 m*M* solution of FITC-myoglobin is made up in 30 m*M* Tris–acetate buffer, pH 5.0. This solution is then fluxed into a naked silica capillary for 100 s (total volume circulated ca. 0.5 ml). Under these conditions, preliminary runs had established that the silica wall was fully saturated with protein. The capillary thus conditioned is then washed for 6 min with the same Tris–acetate buffer, pH 5.0, in order to eliminate all unbound protein and/or additional layers of protein physically bound to the first adsorbed layer. Protein desorption is affected by placing in the cathodic vessel a 25 m*M* phosphate buffer, pH 7.0, containing 60 m*M* SDS (the same buffer, but devoid of SDS, is used as anolyte). The electrophoretic run is performed at 25°C at a voltage drop of 180 V/cm (typical current of 25 μA) in a 24.6 cm (20 cm to the detection window)×100 μm I.D. capillary. Quenching of protein adsorption was obtained via addition of a series of amines, in molarities given under the relevant figures. In this last case, the capillary was first equilibrated with the same pH 5.0 buffer solution, containing the desired amounts of amines, so as to induce any binding of such amines to the capillary wall prior to the exposure to the protein solution. Finally, when the protein solution was flushed into the capillary (100 s) also this last solution contained the same amount of amine used to pre-condition the silica tubing.

2.4. Peak quantitation

The instrumentation used for the experiments was a Bio-Rad (Hercules, CA, USA) Bio Focus 3000, equipped with two laser detectors, one an argon-ion beam (488 nm excitation), the other a helium–neon beam (594 nm excitation wavelength). In order to obtain a precise quantitation of the eluted FITC-myoglobin peaks, the sample was added with an internal standard of sulphorodamine, supplied as a tester kit by Bio-Rad. Thus, within each run, the FITC-myoglobin peak was detected at 520 nm, whereas the internal standard peak was revealed at 630 nm. The internal standard was used for correcting for any variation in peak area resulting from fluctuations in the injected volume.

3. Results

3.1. Preliminary test runs

We have first established a curve of binding of FITC-myoglobin to the naked silica wall as a function of the prevailing pH in solution (from pH 3 up to pH 10). This binding curve was established by the procedures listed in Sections 2.3 and 2.4, with the exception that a series of buffers (at iso-ionic strength) was used titrated at 0.5 pH units increments along the pH scale. The reason why myoglobin was

adopted as a test protein was because it is a fairly small protein (M_r=18 000), it does not have a quaternary structure and it has a p*I* ca. in the middle of the pH scale. As shown in Fig. 1, non-negligible amounts of myoglobin are bound to silica already at pH 3.5; the adsorbed amount increases dramatically as the pH is augmented, it peaks in proximity of the p*I* value and then descends at alkaline pH, but here too it is not extinguished even at pH 10. Curiously, the adsorption curve of myoglobin displays a profile which looks almost like the derivative of the typical sigmoidal curve of zeta potential vs. pH. From this binding profile, it was decided to perform all quenching experiments with the various additives at pH 5.0, as a representative pH of good interaction between a positively-charged macromolecule and a negatively charged silica surface.

Fig. 2 shows a typical electrophoretic desorption profile obtained by adsorbing myoglobin at pH 5.0 and then eluting with the SDS buffer. It is seen that the myoglobin is desorbed as three different peaks, which probably represent moieties with different levels of FITC substituent (FITC, additionally, introduces negative charges on the protein, further contributing to its microheterogeneity). The profile was deliberately printed out of scale, in order to show what happens upon a second electrophoretic desorption with the same SDS buffer: as shown in the bottom tracing, nothing seems to be eluted any more, since the second run does not show any material above the baseline. Fig. 3 shows a typical elution profile of myoglobin desorbed by SDS micelles, run in presence of the internal standard sulphorodamine (the sharp peak with a transit time of ca. 10 min). By this procedure, precise quantitation of material bound to the wall could be obtained. It should be empha-

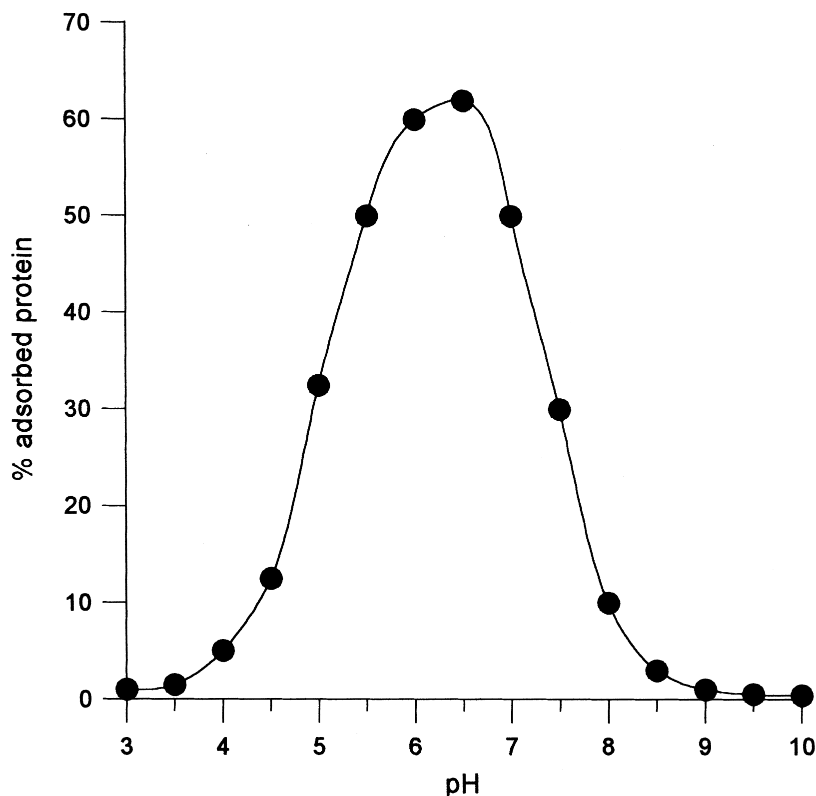

Fig. 1. Adsorption of FITC-myoglobin to a naked capillary surface as a function of the pH of the background electrolyte (at iso-ionic strength). Note that, at pH 5.0, the adsorption can be as high as 40% of the injected protein peak. Here, and in all the following figures, the amount of adsorbed material was calculated from an SDS micelle electrophoretic elution step, as described in Section 2.3; peak quantitation was performed with the aid of an internal standard and a dual-laser beam, as per Section 2.4.

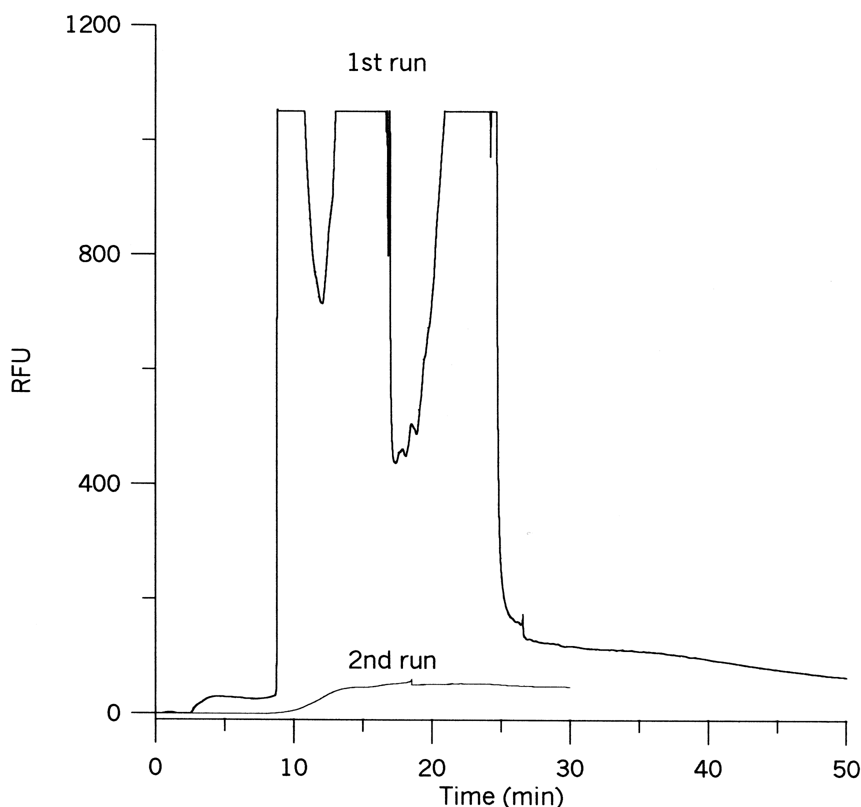

Fig. 2. Typical example of an electrophoretic desorption profile, as elicited by an SDS micelle electrophoretic elution step, after adsorbing FITC-myoglobin at pH 5.0. The lower tracing represents a second SDS elution step; it confirms that no more protein remained bound to the wall, since only baseline fluorescent values can be appreciated.

sised, moreover, that the present study would not have been possible in the absence of fluorescent labelling: the typical amount of protein we desorb are at the pmol level, thus well below UV detection sensitivity.

3.2. Quenching of binding by monoamines

Here, and in the following sections, the amines utilised are listed in Table 1. Fig. 4 compares the efficacy of three amines (triethylamine, ethylamine and ethanolamine) in diminishing the adsorption of FITC-myoglobin to the silica wall as a function of their molarity in solution. The point of maximum adsorption (100%) is taken as the value of adsorption of myoglobin when flushed in the capillary in the absence of any quencher. It is thus seen that the strength of these amines follows this order: triethylamine>ethylamine>triethanolamine. Nevertheless, it should be noted that large amounts (100 to 200 m*M*) of these monoamines have to be added to the background electrolyte in order to ensure a reasonable quenching of protein adsorption. A second phenomenon that should be noted: no concentration can ensure 100% inhibition of protein binding, since all the curves move asymptotically towards the intercept with the *x*-axis. Another way to represent the inhibition strength of each additive is to plot the concentrations which ensures 50% and 90% quenching of adsorption. The first value would represent a kind of dissociation constant, since it would give the molarity of the amine at which the protein would be 50% bound and 50% free in solution (on a relative scale, i.e., by assuming as 100% value only the protein adsorbed to the wall, as done in the present case, and disregarding the protein free in solution; on an absolute scale, this would not be true). The relevant bar graph is shown in Fig. 5,

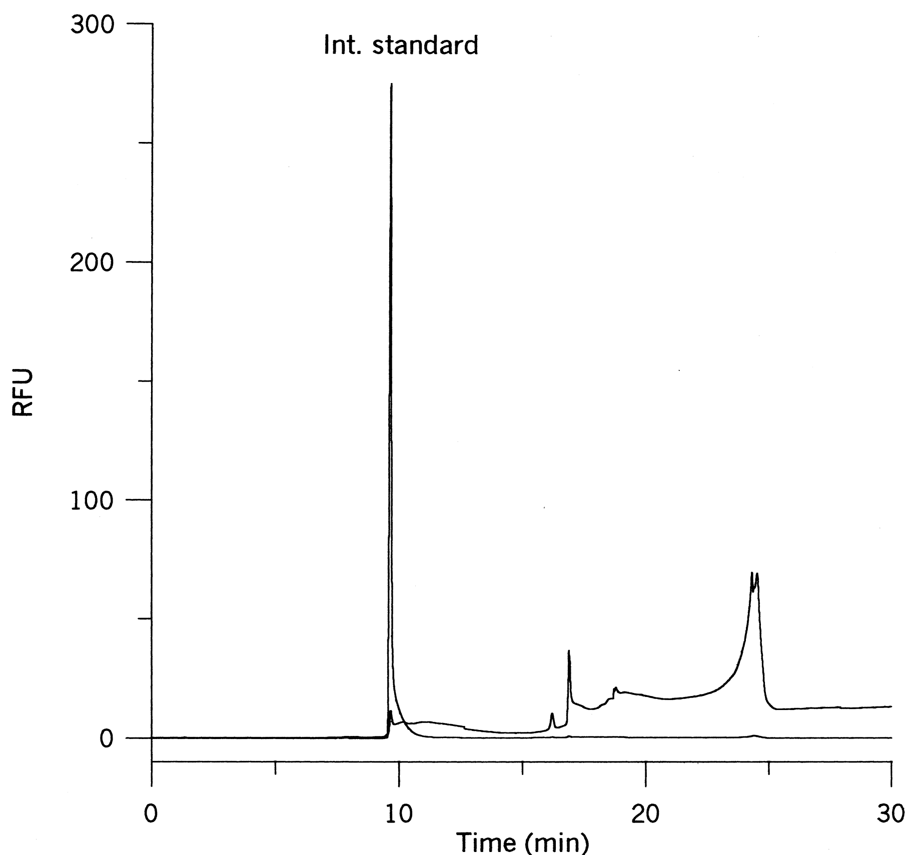

Fig. 3. Typical example of an electrophoretic desorption profile, as elicited by an SDS micelle electrophoretic elution step, after adsorbing FITC-myoglobin at pH 5.0. In this run, the internal (Int.) standard (sulphorodamine) added for precise quantitation purposes, can be seen as a sharp peak eluting at ca. 10 min.

which gives an immediate visualisation of the inhibition power: it is thus seen that whereas 50% quenching is obtained with only 38 m*M* triethylamine, it requires 160 m*M* triethanolamine for obtaining the same effect. The amount of the latter compound able to affect 90% inhibition is terribly large: 560 m*M*, a concentration hardly compatible with a proper electrophoretic experiment, due to the very high conductivity of such a solution.

3.3. Quenching of binding by diamines

A similar set of experiments, obtained with diamines, is shown in Fig. 6: here the efficacy seems to be rather similar, and decreases in the order cadaverine>HMB>putrescine. It should be noted that, although the 50% inhibition values are rather similar (see Fig. 7) and the 90% values substantially lower than those of the monoamines, the range of working values is not dramatically lower than in the case of monoamines. As an example, the 50% inhibition power of triethylamine (38 m*M*) is in fact in the range of values of the three diamines tested. As a general conclusion, it would appear that not much is gained in moving from the mono- to the diamines: the inhibition power is quite similar, which suggests that there is no co-operative effect for a doubly-charged molecule in the binding strength to the silica wall.

3.4. Quenching of binding by oligoamines

The situation changes quite dramatically when adopting oligoamines (see Fig. 8). In this case, only

Table 1
Amines utilised for dynamic coating

Amine	Formula
Ethylamine	H_3N^+
Triethylamine	NH^+
Triethanolamine	HO_2HC NH^+ CH_2OH CH_2OH
Glucosamine	CH_2OH O OH OH OH NH_2
Galactosamine	CH_2OH OH O OH HO NH_2
Putrescine	H_3N^+ NH_3^+
Cadaverine	H_3N^+ NH_3^+
Hexamethonium bromide	N^+ Br^- N^+ Br^-
Spermidine	H_3N^+ NH NH_3^+
Spermine	H_3N^+ NH NH NH_3^+
TEPA (tetraethylenepentamine)	H_2N NH NH NH NH_2

spermidine (which is a triamine) still shows a need for substantial concentrations. The other two amines (spermine, a tetramine, and TEPA), when plotted on the same scale as spermidine, show a drastic decrease in concentration, in the range of sub-millimolar. As shown in Fig. 9, the quenching strength decreases as follows: spermine>TEPA>spermidine. It is quite remarkable that spermine (a tetramine) should show an inhibition power (at 50% level) more than one-order of magnitude better than TEPA,

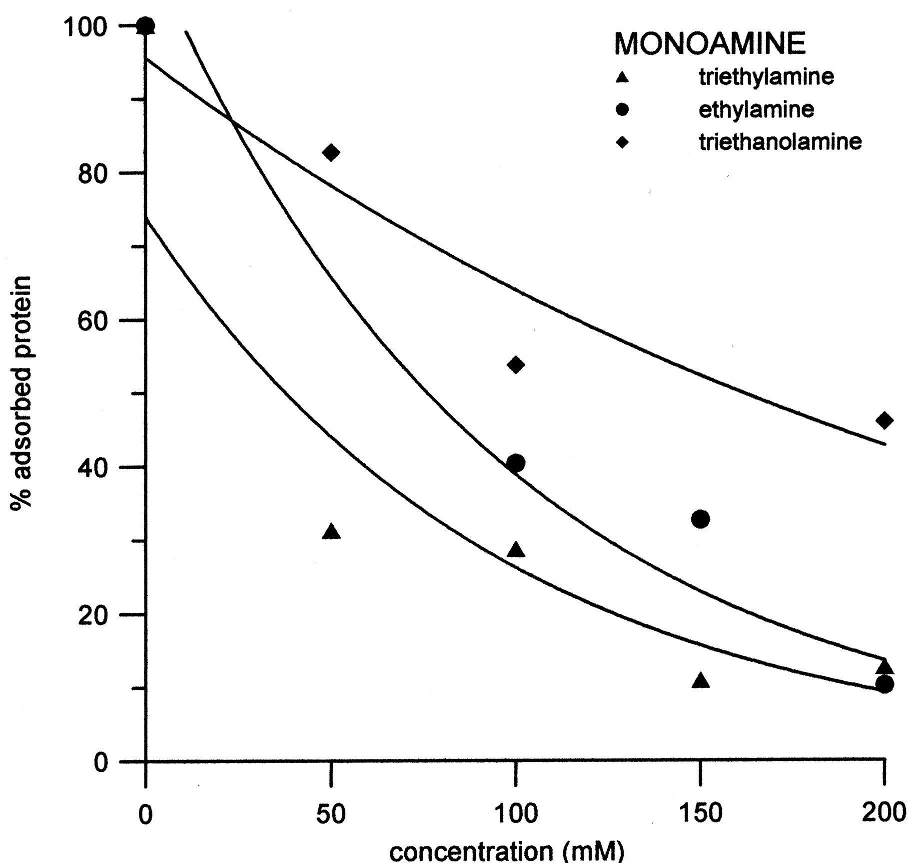

Fig. 4. Quantitation of the efficacy of three monoamines (triethylamine, triangles; ethylamine, circles and triethanolamine, diamonds) in inhibiting FITC-myoglobin binding to the silica wall, as a function of their respective molarities in the background electrolyte (30 m*M* Tris–acetate, pH 5.0).

although the latter is a pentamine. In turn, TEPA also exhibits an inhibition strength more than one-order of magnitude better than spermidine. As a result of these findings, it would appear that either spermine or TEPA can be added to any background electrolyte at such minute concentrations so as not to alter the conductivity and ionic strength of the background electrolyte.

3.5. On the efficacy of different washing procedures

The present technique, since it enables us to fully quantify protein adsorption, has permitted us a proper evaluation of the efficacy of different conditioning procedures for eliminating proteinaceous material adsorbed to the wall. Up to the present, such washings have been performed by approximation, without really knowing or properly monitoring the state of the silica wall. Thus, in almost all published procedures, a typical sequence of washing steps is to first treat with 1 *M* NaOH, followed by abundant rinsing, then to treat with 1 *M* HCl, again followed by generous rinsing in distilled water and final conditioning of the capillary in the running buffer. We have devised the following: since we know that our SDS micelle procedure is effective in eliminating essentially 100% of any proteinaceous material bound to the wall, we have saturated the silica with protein at pH 5.0, as described, then washed the capillary either with 1 *M* NaOH or with 1 *M* HCl. At the end of these steps, we would wash the silica wall

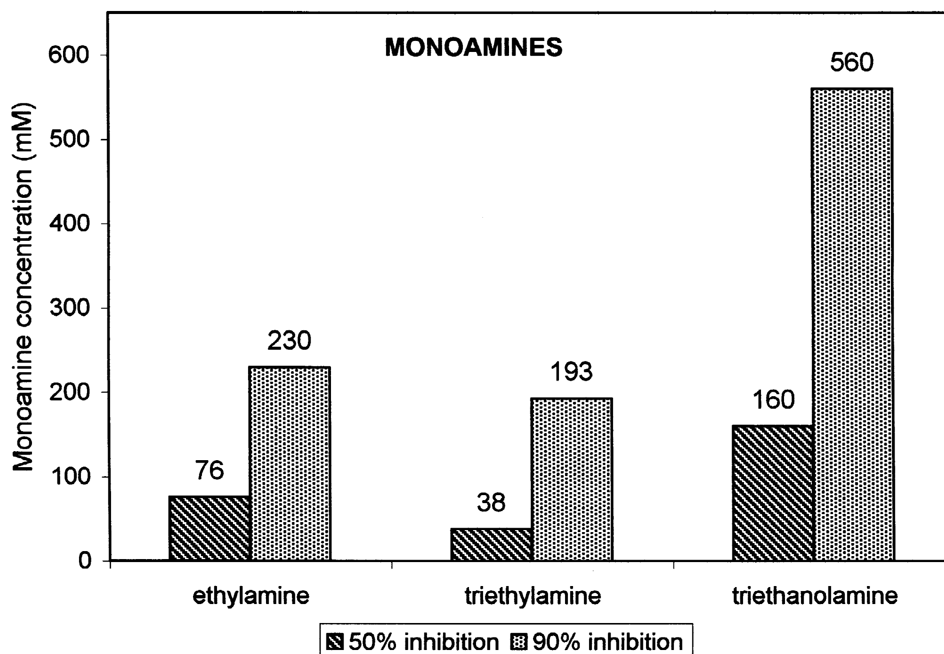

Fig. 5. Figures of merit for the binding inhibition of the three amines of Fig. 4. The two bars for each compound represent the molarites eliciting 50% (diagonal shadings) and 90% (dot fillings) inhibition of binding of FITC-myoglobin to the naked silica, when added to a background electrolyte composed of 30 m*M* Tris–acetate, pH 5.0.

again with an additional sweeping step of SDS buffer. Sure enough, as illustrated in Fig. 10, material which had not been completely released from the wall now emerged in the SDS buffer. Although the amount of protein desorbed with this last step is very minute (it amounts to only 0.05% and 0.1% of the initial protein load in NaOH and HCl rinsings, respectively) this shows that neither washing procedure is appropriate in releasing all the protein from the wall and that, in any event, the best procedure between the two is still a washing step in NaOH. The reverse did not apply, of course: i.e., when the silica surface was treated first with SDS micelles, followed by either an NaOH or an HCl rinsing, no additional protein material could be desorbed (not shown). The implications of these findings will be further discussed below.

4. Discussion

Our findings (we believe the first ones dealing with real quantitation of the adsorption and desorption phenomena) have some very important practical implications. First of all, it should be noted that never before has an evaluation of this kind been attempted: typically protein binding (or the presumed absence of binding) was evaluated via a set of visual parameters on the electropherogram: peak shape, absence (or presence) of tailing, improved resolution and the like. Although these parameters are surely helpful in assessing the merits of each amine quencher, they could not possibly give figures of merit as deducible from our data. It must be stated that, in fact, there was a single, serious attempt at evaluating in a quantitative manner this phenomenon; it came from Regnier and Lin, who devised an instrument containing two detectors, placed 50 cm apart [2]. The decrement of peak area, as measured at the second detector, would give unambiguously the amount of protein adsorbed. This method was only used, however, precisely for measuring protein adsorption along the pH scale, without any aim at quantifying or investigating any desorption phenomenon. It was thus found, as an example, that most basic proteins (chymotrypsinogen, ribonuclease A, cytochrome *c*

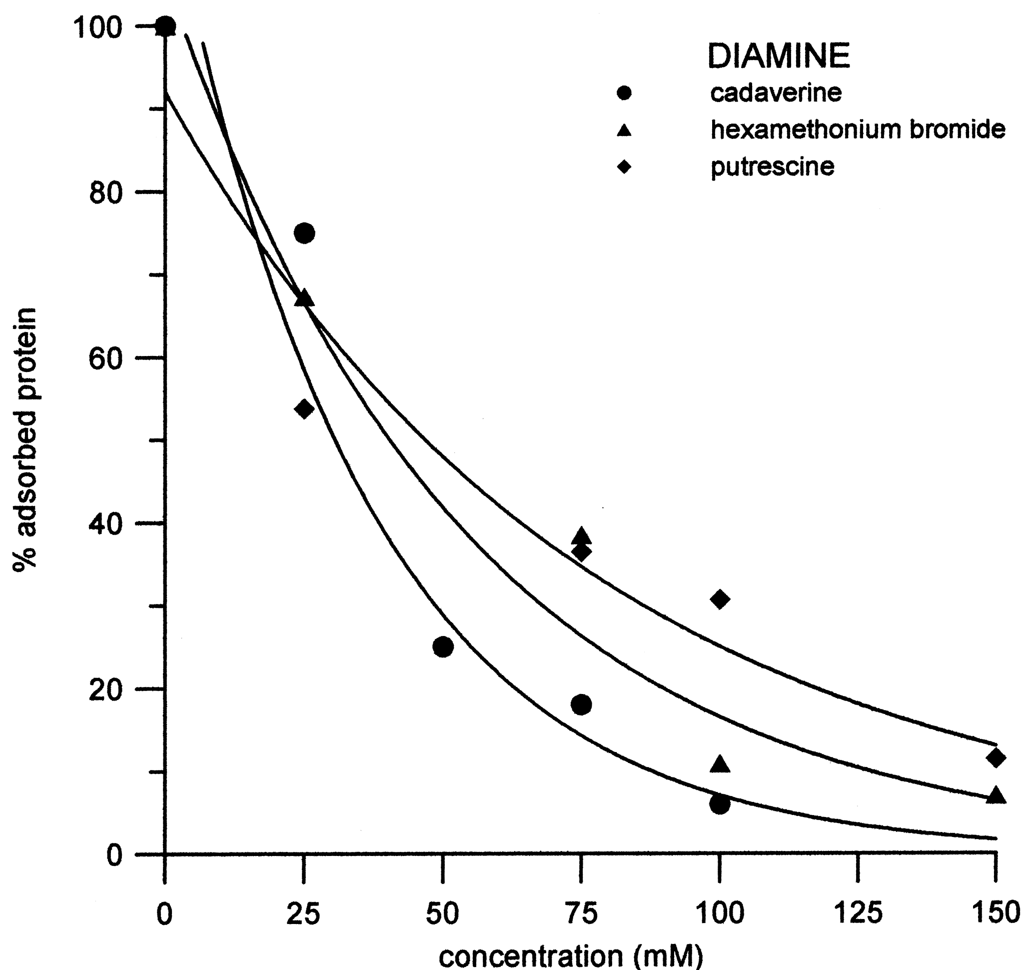

Fig. 6. Quantitation of the efficacy of three diamines (hexamethonium bromide, triangles; cadaverine, circles and putrescine, diamonds) in inhibiting FITC-myoglobin binding to the silica wall, as a function of their respective molarities in the background electrolyte (30 m*M* Tris–acetate, pH 5.0).

and lysozyme) were 100% adsorbed onto the silica wall in the pH 7–8 range. In addition, this method would not be easily available as a routine procedure in a research laboratory, since it would require extensive modification of commercial instrumentation.

The present investigation clearly shows that not much is gained, in discouraging protein adsorption to the silica wall, by using mono- or diamines as additives to the background electrolyte. When analysing the bar graphs of Figs. 5 and 7, one can notice, in fact, that, in order to elicit 90% inhibition of adsorption, one has to use exaggerated levels of amines, ranging from 200 to 560 m*M* for the mono-, to 100 to 170 m*M* for the diamines. The concomitant increments in ionic strength of the background electrolyte would result in too high currents and would thus require rather low voltage gradients, which in turn would be detrimental to the separation process. It is of interest to speculate on the mechanism of quenching of such interactions, which must rely primarily on the binding of the amine themselves to the wall, this preventing or minimising protein binding. It seems logical to assume that the amines are first attracted to the silica surface by Coulombic forces and, once there, are further re-

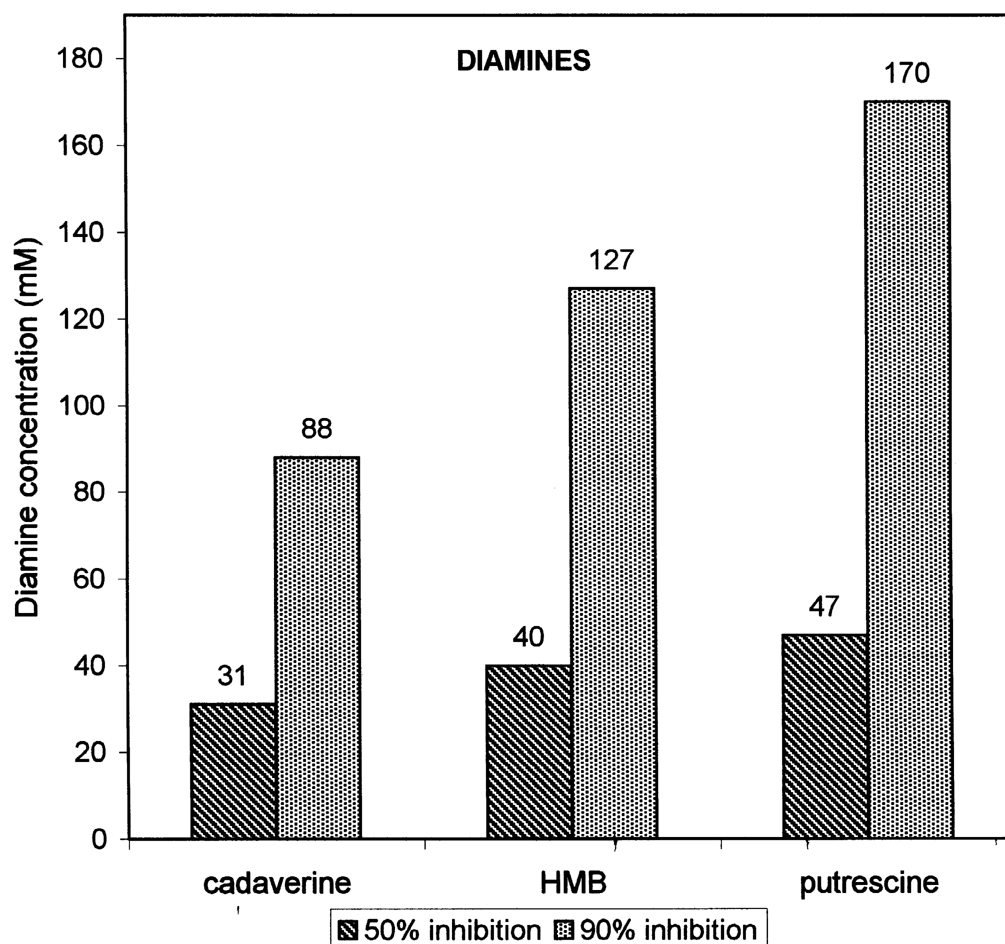

Fig. 7. Figures of merit for the binding inhibition of the three amines of Fig. 6. The two bars for each compound represent the molarites eliciting 50% (diagonal shadings) and 90% (dot fillings) inhibition of binding of FITC-myoglobin to the naked silica, when added to a background electrolyte composed of 30 m*M* Tris–acetate, pH 5.0.

tained by additional mechanisms, such as hydrogen bonding and perhaps hydrophobic interactions (all these events might even occur simultaneously). The latter mechanism might not be far from reality: it is noted, for instance, in Fig. 5, that the order of efficacy follows the order of hydrophobicity of these compounds: thus triethylamine, the most hydrophobic of the series, is much more effective than triethanolamine, the most hydrophilic species. If hydrogen bonding were the main retaining mechanism, the order should be reversed: triethanolamine, by virtue of possessing three –OH groups, should be more extensively hydrogen bonded to the wall than triethylamine. When referring to the oligoamines, the mechanism could be more complex: it seems to be quite clear that here the main driving mechanism of their interaction with the wall could rest mainly on the number of charges, which, above a critical number, could act co-operatively. In fact, there is a drastic increment of efficacy in going from a triamine (spermidine) to tetra- (spermine) and penta- (TEPA) amines. Here too there are some subtle but important differences, as shown in Fig. 9: the most efficient quencher, by far, seems to be spermine and not TEPA. There could be two reasons for this: first of all, due to the proximity of the amino groups, the p*K* values of TEPA drastically diminish along the oligoamino backbone: these values are given as

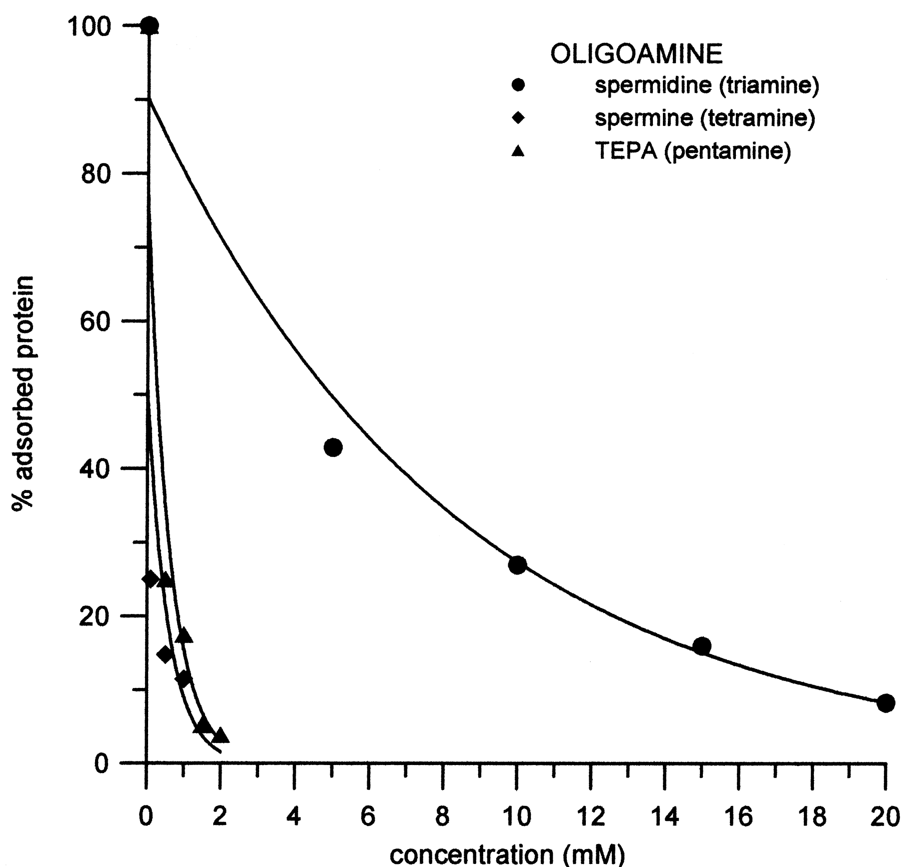

Fig. 8. Quantitation of the efficacy of three oligoamines (TEPA, triangles; spermidine, circles and spermine, diamonds) in inhibiting FITC-myoglobin binding to the silica wall, as a function of their respective molarities in the background electrolyte (30 m*M* Tris–acetate, pH 5.0).

(from pK_1 to pK_5: 9.9, 9.1, 7.9, 4.3 and 2.7) [45]. Thus, at the operative pH of 5, this molecule will have, on the average, 3.5 positive charges. On the contrary in spermine, where the nitrogens are spaced three carbon atoms apart at the two extremes of the molecule, and four carbons apart in the middle, the p*K* values are still located in the alkaline region, which means that the effective charge of spermine is higher than that of TEPA. But here too an additional mechanism of hydrophobic interaction cannot be excluded: spermine, containing segments of three to four carbons (as opposed to two for TEPA) in between the nitrogens, should have a somewhat higher hydrophobicity than TEPA and thus could interact even more strongly with the silica wall. That this could be the case is further supported by observations made by Regnier and Lin [2]: these authors have evaluated the efficacy of amines on the basis of two parameters, the CH_2/NH ratio and the molecular mass. In the case of spermine, this ratio is 2.5 (10/4), whereas it is lower (8/5=1.6) in TEPA. Moreover, even the molecular mass of spermine (202 g/mol) is higher than that of TEPA (192 g/mol), this additionally confirming the higher efficacy of the former molecule.

A final comment should be made on the power of the various conditioning procedures for cleansing the capillary wall. Typical washings, generally accepted in most laboratories, consist in treating the capillary either with 1 *M* NaOH, or with 1 *M* HCl, or with both. Although these steps might be quite adequate for general purposes and in the case of small

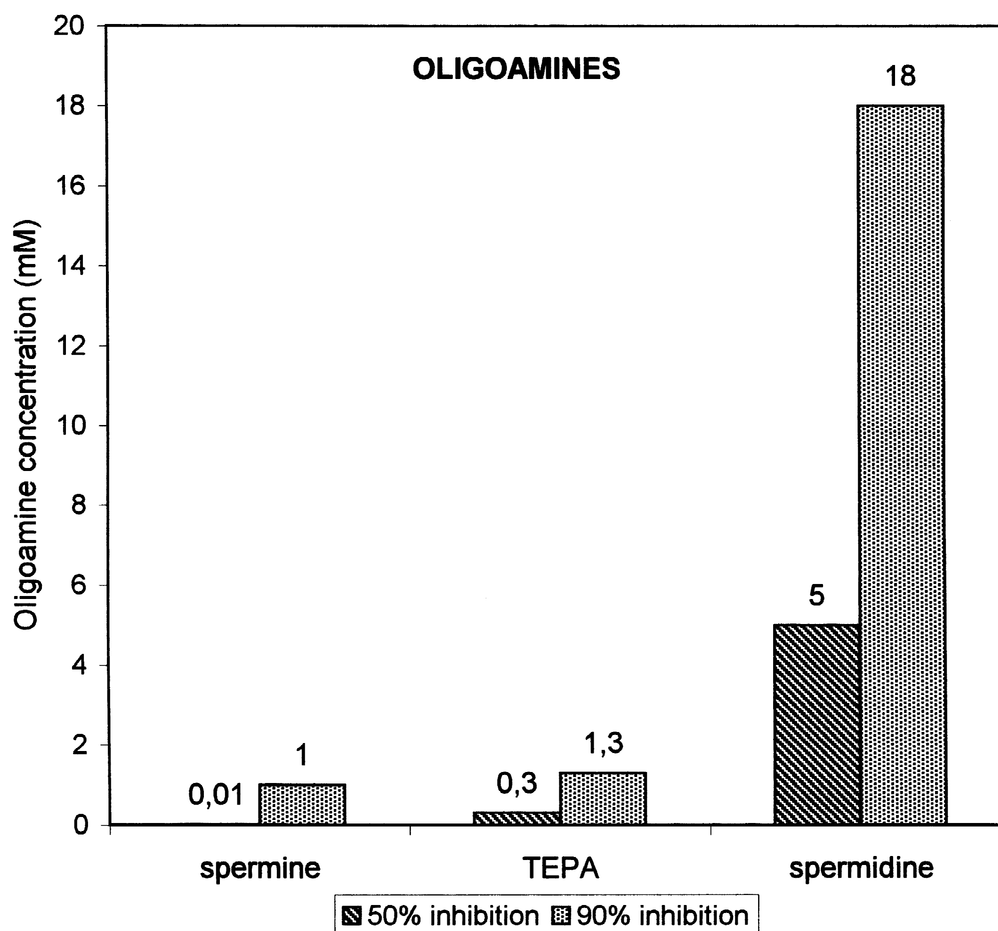

Fig. 9. Figures of merit for the binding inhibition of the three oligoamines of Fig. 8. The two bars for each compound represent the molarites eliciting 50% (diagonal shadings) and 90% (dot fillings) inhibition of binding of FITC-myoglobin to the naked silica, when added to a background electrolyte composed of 30 m*M* Tris–acetate, pH 5.0. Note that for TEPA and spermine these values are in the sub-millimolar range.

metabolites, they do not seem to be as efficient in protein desorption, as shown in Fig. 10. Only a rinsing step consisting in driving SDS micelles into the capillary lumen seems to be efficient in releasing essentially 100% of proteinaceous material. Washings with HCl or with NaOH seem to leave behind traces of macromolecules still bound to the wall, although the NAOH conditioning step seems to be more efficient than the HCl treatment. This could have important practical implications. It has been demonstrated long ago [46] that these cycles of conditioning the wall with NaOH, followed by HCl (or vice versa) leave the silica surface in a state of total chaos: the silanols undergo a profound hysteresis loop before returning to their original ionization state; a loop which could take as long as two weeks for termination. This should not happen with the SDS washings, since this procedure is performed at neutral pH; in addition, upon washing in water, the micelles should disaggregate and the free SDS should leave the capillary lumen.

Acknowledgements

Supported by grants from MURST (Coordinated

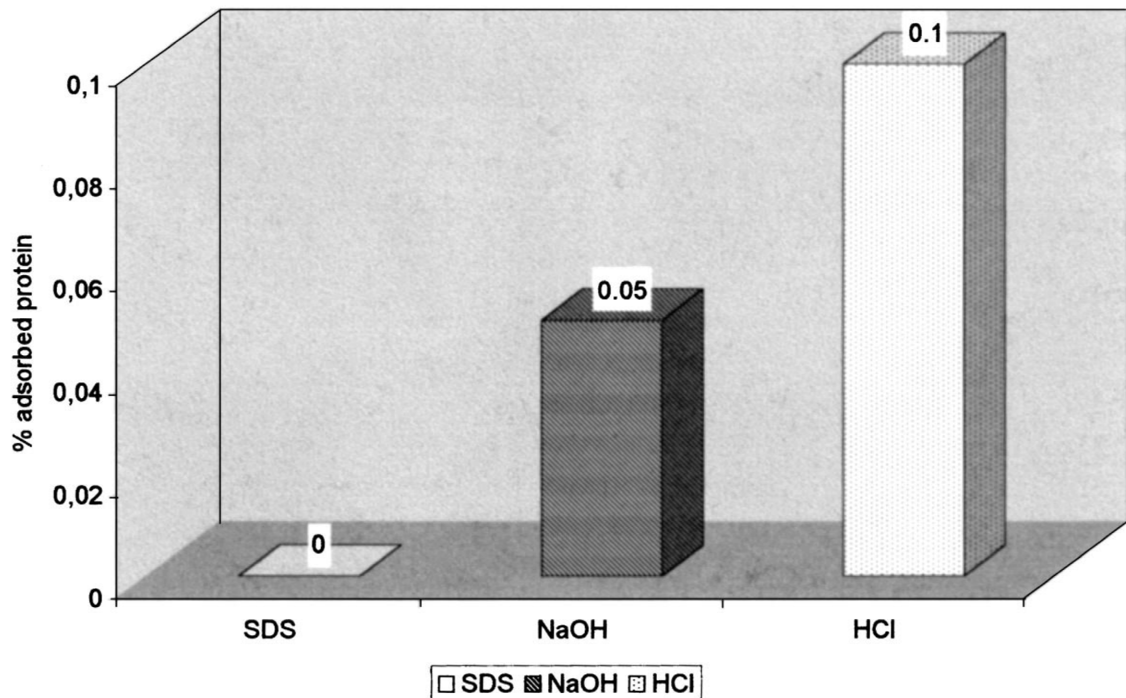

Fig. 10. Efficacy of different washing steps in desorbing bound protein to the silica wall. Washings (from left): 60 m*M* SDS in 25 m*M* phosphate buffer, pH 7.0 (electrophoretic desorption); 1 *M* NaOH for 6 min.; 1 *M* HCl for 6 min. Note that only the SDS step ensures 100% removal of bound protein from the wall.

Project Protein Folding Misfolding, 1999) and from ASI (Agenzia Spaziale Italiana, Rome), grant No. ARS-99-22.

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