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p-Toluenesulfonyl Chloride as an Activating Agent of Agarose for the Preparation of Immobilized Affinity Ligands and Proteins. Optimization of Conditions for Activation and Coupling

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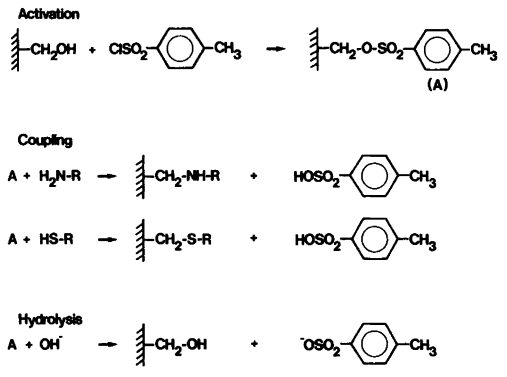
A new immobilization technique suitable for the binding of biomolecules to polysaccharide supports, such as agarose beads, is described. It involves treatment of agarose with *p*-toluenesulfonyl chloride (tosyl chloride) with the formation of *p*-toluenesulfonic ester (tosylation). This reaction is rapid and can be controlled to give a range of substitutions up to very high levels. The tosylated agarose obtained shows excellent long-term stability and the swelling properties of the gel are similar to those of untreated agarose indicating that no cross-linking of the support occurs during tosylation. The degree of substitution of agarose is easily monitored by UV measurement of the appearance of *p*-toluenesulfonate. Addition of nucleophiles, such as amino or mercapto group-containing compounds, leads to efficient displacement of the tosyl groups. With β-alanine and β-mercaptopropionic acid as model compounds the degree of coupling was investigated as a function of time, temperature and pH. High degrees of substitution (in the range 6 – 10 μmol/g wet gel) were obtained within 10 h at pH 8 – 10 and room temperature. Higher yields were obtained on prolonged reaction time, more alkaline conditions, and higher temperature. It was concluded from titration data that ligands were coupled without the introduction of any additional charged groupings and that on coupling of thiol compounds uncharged linkages were formed between ligand and support. Coupling in organic solvent of ligands not soluble in water gave high yields of coupled product. Some results of the coupling of biomolecules, such as affinity ligands and enzymes, to agarose are given.

A number of immobilization techniques leading to covalent attachment of biomolecules, such as affinity ligands[1] or enzymes,[2] are known and applied. Probably the most common procedure for binding to polysaccharide supports involves activation by cyanogen bromide,[3] while another frequently used polysaccharide support carries epoxy groups.[4] Although these procedures have proved successful, they may have drawbacks under certain conditions (see Results and Discussion).

We, therefore, feel that the more coupling methods that are available the better the chances of finding an immobilization procedure tailor-made for a particular purpose. We have found tosylated polysaccharides to be suitable alternative supports for immobilization. It is known from the chemistry of soluble saccharides that reaction of hydroxyl groups with *p*-toluenesulfonyl chloride (tosyl chloride) forms the corresponding esters (tosylates) in which the tosyl groups have excellent leaving properties in reactions with nucleophiles giving stable linkages between ligand and saccharide carbon.[5,6] This finding



Scheme 1.

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prompted us to try to develop a procedure suitable for the binding of biomolecules to polysaccharides. The pathway of these reactions are analogous to those of tosylates of soluble saccharides [5,6] and are throught to involve the steps given in Scheme 1.

In this study the chemistry involved is discussed together with investigations of various variables playing a role in ligand binding.

EXPERIMENTAL

N,N-Dimethylformamide (DMF, spectroscopic grade), diglyme (diethyleneglycol dimethyl ether) *p.a.*, acetone *p.a.*, glycerol (87 %) *p.a.*, dried dioxane (max. 0.01 % H_2O), hexylamine *p.s.* and *p*-toluenesulfonic acid *p.a.* were from Merck, Darmstadt, West Germany. Sepharose 6B and Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Histamine hydrochloride (98 %) and *p*-toluenesulfonyl chloride (98 %) were from Aldrich-Europe, Beersee, Belgium. Pyridine was bought from BDH Chemicals Ltd., Poole, England. β-Alanine, γ-aminobutyric acid (99 %) and 1,6-hexanediamine were from Sigma Chemicals, St. Louis, Mo., U.S.A.

ε-Aminocaproic acid (99 %) was from Kebo AB, Stockholm, Sweden, β-mercaptopropionic acid (99 %) from Fluka AG, Buchs, Switzerland, Omniflour from New England Nuclear, Albany St., Boston, Mass., U.S.A. and Triton-X-100 (scintillation grade) from Eastman Kodak Company, Rochester, N.Y., U.S.A. (Ring−2−^{14}C)-histamine dihydrochloride (2.2 GBq/mmol in 0.25 ml) was from the Radiochemical Centre, Amersham, U.K. All other chemicals were of analytical grade and used as supplied. Redistilled water was used throughout.

Agarose gel beads. Gels were washed on a glass filter funnel and equilibrated in each washing medium for some minutes before the suspensions were gently sucked dry by vacuum until a wet gel cake was obtained (care was taken to avoid drawing air into beads). In order to determine the degree of swelling of the gel beads they were weighed, freeze-dried for about 24 h and then reweighed.

Activation (tosylation). The following procedure holds for all activations and can be scaled up or down if needed. When the reaction was done in diglyme or acetone we used the same procedure as that described below. Unless otherwise stated, the dioxane used in the washings was of analytical grade.

Wet Sepharose CL-6B was washed with 2 × 10 gel volumes of water, water−dioxane 3:1 (v/v), water −dioxane 1:3, dioxane and finally with 3 × 5 gel volumes of dried dioxane (containing less than 0.01 % water). The purpose of this washing procedure was to get rid of water in accordance with similar washing procedures [7] (water may hydrolyze the tosylating agent, tosyl chloride).

The above Sepharose (7 g wet weight) was then transferred to a round-bottomed flask containing 1 g of tosyl chloride dissolved in 2 ml of dried dioxane. With magnetic stirring 1 ml of pyridine was added dropwise for about 1 min. After 1 h at room temperature, the gel was washed with 2 × 10 gel volumes of dioxane and then gradually transferred back to water by reversing the washing scheme described above. The gels were stored in distilled water at 4 °C until used.

In all tosylations the same 1:1 ratio (w/v) of tosyl chloride to pyridine as that used above was applied. Preparations A and B were made by taking aliquots after 10 and 30 min, respectively, from the reaction mixture and washing them immediately with dioxane, as described above. Preparation G was made in exactly the same way as preparation C, using Sepharose 6B.

Coupling procedures. The following procedure was used for coupling amino or mercapto group-containing ligands: The ligand was dissolved in 0.5 ml of 0.5 M sodium bicarbonate buffer of pH appropriate for coupling (see Results and Discussion section for further details) and kept at 4 °C. A wet tosylated agarose preparation was washed with the above cold bicarbonate buffer, gently sucked dry, after which 1.75 g was added to the solution with dissolved ligand. The pH was checked and corrected, if necessary, by addition of 3 M NaOH or 3 M HCl. The mixture was placed in a water-bath with a shaker for coupling at 40 °C. A rocking table was used when coupling was carried out at 4 or 20 °C. After coupling the support was washed with 4 × 10 gel volumes of the following: Distilled water, 1 mM HCl, 1 M Na_2CO_3, distilled water.

Hexylamine was coupled in DMF by dissolving 0.16 ml of hexylamine in 0.34 ml of DMF and adding 1.75 g wet tosylated agarose (preparation C), which had first been carefully washed free from water with DMF. Coupling was done in a water-bath with a shaker at 40 or 60 °C. The products were carefully washed with DMF and twice with acetone in order to wash away unbound ligand.

UV-measurements and determination of tosyl group content. Spectra were recorded between 320 and 200 nm with a Beckman double-beam spectrophotometer in cuvettes with 1 cm light path. A glycerol−water solution (87:13 w/v) was used to get stable and homogeneous gel suspensions. To the sample and reference cuvettes were added 100 mg of the wet gel to be analyzed and untreated Sepharose CL-6B gel, respectively.

To each cuvette was added 2.9 ml of 87 % glycerol −water solution. The cuvettes were mixed for 3−4 min or until the suspensions were homogeneous. The absorption spectra were then recorded at a scanning

speed of 2 nm s^{-1}. 2–3 spectra were taken and the mean value was used. The extinction coefficient for ethyl tosylate at 261 nm, 480 M^{-1} cm^{-1},[8] was used to calculate the tosyl group content in wet tosyl gels.

When analyzing the hydrolysate from 1 M NaOH treated tosylated gels, the UV spectra were taken with 1 M NaOH as reference. To calculate the amount of *p*-toluenesulfonic acid in the hydrolysate, the extinction coefficient at 260 nm for the acid in 1 M NaOH was determined from absorption spectra on standard solutions of the acid. The absorption spectra of *p*-toluenesulfonic acid was practically the same in 87 % glycerol, distilled water and 1 M NaOH.

The sulfur, nitrogen and chloride contents of freeze-dried tosylated gels were determined by elemental analysis. Gels washed as described under "Activation" and gels which had also been washed carefully with chloroform and acetone were analyzed. The tosyl group content was calculated from the sulfur content and the amount of tosyl groups per disaccharide subunit (mol. wt. 306) was then calculated.

Determination of the amount of coupled ligands. The amount of immobilized ligands was determined by titration. In addition, elemental analysis (on hexylamine preparations and on some of the β-alanine preparations) and radioactivity measurements (of histamine preparations only) were also done. The degree of swelling was determined as described above and it was found that the swelling of the tosylated preparations was not significantly changed after coupling of ligands.

Titration was done with 0.2 M NaOH at a low speed (0.25 μl s^{-1}) in a titration assembly with automatic recording of the titration curve. The gels to be analyzed were washed in 1 M KCl on a glass filter funnel, as described under "Agarose beads". To 1 g of the wet gel, placed in a titration vessel, appropriate volumes of 1 M KCl and 1 M HCl were added to get the desired titration volume and starting pH, respectively. Titrations were carried out at a pH ranging from 2.0 to 11.5.

Immobilized histamine was determined by radioactivity measurements of the hydrolysate of the freeze-dried preparations, as described by Nilsson and Mosbach.[9]

RESULTS AND DISCUSSION

Activation (tosylation) of agarose. The activation was achieved mainly with dioxane as solvent, but later we found diglyme (diethyleneglycol dimethyl ether) and acetone to be good alternatives. In order to get high yields of tosyl groups on the polymer, conditions as water-free as possible had to be used during tosylation, since water rapidly hydrolyzes the tosyl chloride. We therefore used dried dioxane (containing less than 0.01 % water) as solvent to get constant conditions and reproducible results.

Table 1 gives the variation of the amount of tosyl groups incorporated per g of dry gel with the amount of tosyl chloride and reaction times used in tosylation. The amount of tosyl groups was determined by elemental analysis of the sulfur content of the different freeze-dried gels. It is clear from Table 1 that on application of 2.5 g tosyl chloride g^{-1} dry agarose (=17.5 ml wet), the reaction is fairly rapid. After only 10 min as much as 0.62 mmol tosyl groups were introduced per g dry product. Extending the contact time to 60 min gave a product with 1.15 mmol tosyl groups g^{-1}. This is a high degree of substitution, higher than the oxirane content obtained after 8 h reaction of Sepharose 6B with 1,4-butanediol diglycidyl ether under optimal conditions (about 1 mmol g^{-1} dry gel),[4] and it is

Table 1. Tosylation of agarose. For all preparations Sepharose CL-6B was used except for sample G where Sepharose 6B was used. Calculations were done as described under Experimental.

Preparation	Tosylation time min	Amount of tosyl chloride used in tosylation g g^{-1} of dry agarose	Found S % (w/w)	Amount of tosyl groups in product mmol g^{-1} of dry weight	Number of tosyl groups per disaccharide unit
A	10	2.5	2.01	0.62	0.21
B	30	2.5	3.01	0.94	0.34
C	60	2.5	3.70	1.15	0.43
D	60	5.0	4.83	1.51	0.60
E	60	1.25	1.98	0.62	0.21
F	60	0.5	0.60	0.19	0.06
G	60	2.5	3.29	1.03	0.38

much higher than the substitution of commercially available CNBr- or epoxy-activated preparations (0.4 mmol cyanate ester groups g^{-1} dry gel[10] and 0.3–0.4 mmol epoxy groups g^{-1} dry gel,[11] respectively).

A fairly linear increase of the amount of tosyl groups in the product was obtained on increasing tosyl chloride concentrations to 2.5 g g^{-1} dry gel.

The tosylation reaction is known to have marked selectivity for primary hydroxyls in sugars.[5,12] The repeating disaccharide subunit in agarose, consisting of 3-linked D-galactopyranose and 4-linked 3,6-anhydro-L-galactopyranose, with a molecular weight of 306, contains one primary hydroxyl when the polymer is not cross-linked.[13,14] In preparation D with 1.5 mmol of tosyl groups g^{-1} gel, this would mean that over 60 % of the original primary hydroxyls were tosylated (compare with Table 1). Since we have normally used cross-linked agarose the percentage of substituted hydroxyl groups was even higher.

Owing to the high stability of the cross-linked agarose used, the degree of swelling of all the gels remained almost constant and was roughly equivalent to that of the original agarose preparation. The only exception was the highly substituted preparation D, which swelled about 10 % less than the lower substituted gels calculating from the agarose content in the products. This was probably due to its high level of hydrophobic groups, which are known to cause shrinkage of agarose gels in water.[15] (Almost 25 % of the dry weight was made up of tosyl groups).

To get optimal coupling of amines it was not necessary to use more than 2.5 g tosyl chloride g^{-1} dry gel in the tosylation giving 1.15 mmol of tosyl groups g^{-1} dry gel (Fig. 1). This amount of reactive groups is in agreement with the epoxy method where about 1 mmol epoxy groups g^{-1} is required for optimal yields.[4]

β-Mercaptopropionic acid was, as expected, more reactive than β-alanine. The former required gels of lower activation level and it was not necessary to activate with more than 1.25 g tosyl chloride g^{-1} dry gel to get good binding.

To ascertain whether non-cross-linked agarose can be tosylated by the method described above, Sepharose 6B was allowed to react with tosyl chloride. As seen in Table 1 (preparation G) the degree of tosylation was high. No optimization has been carried out. In one experiment, β-mercaptopropionic acid was coupled to the activated support and the amount bound was 12 μmol g^{-1} wet preparation (coupling done at pH 10, 40 °C for 40 h with 0.4 M ligand) and should be compared with 18 μmol with Sepharose CL-6B under similar conditions. Other commercially available agarose preparations, such as Sepharose 4B, were not studied in this investigation. They too can probably be activated by the method described here.

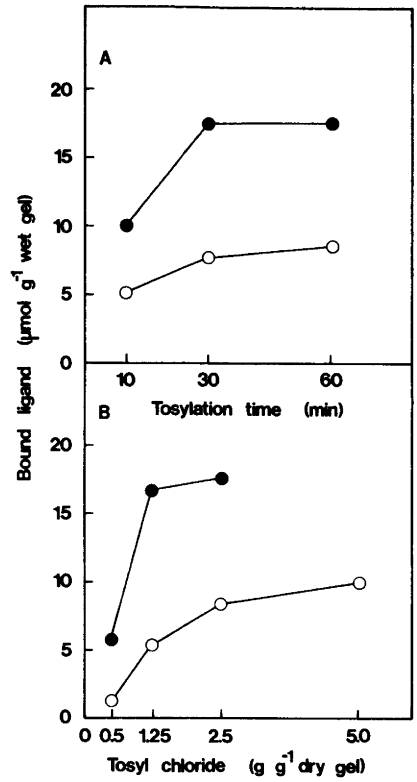

Fig. 1. Coupling of β-alanine (○) and β-mercaptopropionic acid (●) to different tosyl Sepharose CL-6B preparations as made when using (A) different times for tosylation and (B) different amounts of tosyl chloride during tosylation. In (A) 2.5 g tosyl chloride g^{-1} dry Sepharose was used and the tosylation time was 60 min in (B). Coupling was carried out for 20 h at 40 °C as described under Experimental with 0.4 M ligand at pH 10.6 (β-alanine) and pH 10.0 (β-mercaptopropionic acid). The amount of bound acid was determined by titration.

UV measurements on tosyl gels. The UV spectra of tosyl chloride, -acid and -esters show characteristic

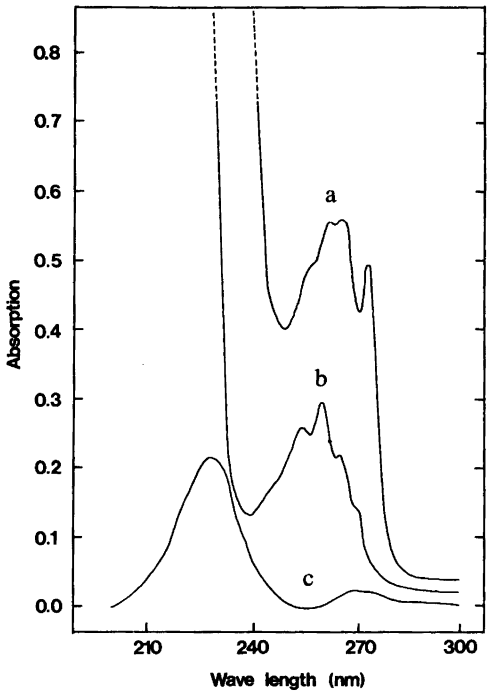

Fig. 2. UV spectra of (a) preparation A from Table 1, (b) the hydrolysate from 1 g wet gel (= 50 mg dry) of sample A and (c) preparation A after treatment with 1 M NaOH for 60 h at 60 °C. The total volume of the hydrolysate was 50 ml and spectra were taken directly on the hydrolysate in a double-beam spectrophotometer using 1 M NaOH as reference. Spectra on the gel samples were taken as described under Experimental.

absorption maxima.[8] UV measurements were therefore used to confirm that the sulphur content determined by elemental analysis was that of the tosyl ester. Ethyl tosylate taken as an example of the latter has a spectrum differing from the chloride and acid and has absorption maxima at 232, 261 and 273 nm, minimum at 249 nm and has a shoulder at 267 nm. The same maxima and minimum were also found for the spectra of the tosylated gels indicating formation of tosyl esters. Also, the relative intensities of the maxima at 273 and 261 nm and of the minimum at 249 nm were similar in the tosylated gel and in the ethyl tosylate spectra.

The spectrum of a tosyl gel, preparation A of Table 1, is given in Fig. 2 (curve a). This figure also gives the spectrum of the filtrate (curve b) obtained on hydrolysis of preparation A, as well as the spectrum of the gel remaining after hydrolysis (curve c).

The spectrum of the hydrolysate from the gel corresponds to that of *p*-toluenesulfonic acid. The amount of acid in the hydrolysate was calculated and found to correspond with the tosyl content given in Table 1 based on elemental analysis of sulfur.

As can be seen in Fig. 2, nearly all the original tosylate groups on the gel were hydrolyzed. Elemental analysis of the gel indicated that about 0.1 % (w/w) sulfur remained on the support. Absorption measurements at 261 nm can be used for the determination of the tosyl content of the gels. Fair correlation (± 5 %) with the elemental analysis were found except for the very highly substituted gels (preparation C and D) where flattening of the spectra occurs resulting in about 15 and 30 % lower values than obtained with elemental analysis. (Such flattening of the absorption spectrum of strongly absorbing particles has been described by Duysens.)[16]

To check further that the sulfur was not to any great extent due to adsorbed *p*-toluenesulfonic acid, the tosyl gels were carefully washed with acetone and chloroform and analyzed for sulfur again: The decrease in sulfur was less than 5 %. Because of this, and the above UV measurements, it was concluded that the sulfur content determined by elemental analysis corresponded almost entirely to tosyl ester.

The gels were analyzed for nitrogen and chloride to ascertain whether any of the side reactions most frequently reported for soluble saccharides, such as displacement of tosyl by pyridine or chloride, had occurred during activation.[5] At most, traces were found, indicating that these reactions do not take place to any significant extent. Thus, unlike the CNBr and epoxy methods (giving unreactive carbamates[2] and cross-linking,[4] respectively), the activation procedure described here does not seem to lead to unwanted side-effects.

Stability of tosyl gels. The tosyl preparations were very stable when stored in the cold in the wet state in distilled water and could be used as required. Preparation C, for example, showed a 25 % decrease in the coupling yield of β-mercaptopropionic acid (coupling done at 40 °C for 15 h at pH 10 with 0.4 M ligand) after storage in the cold for 3 months in distilled water. It is probable that storage under slightly acidic conditions (pH 4 – 5) or freeze-drying would render the preparations even more stable.

The stability of the tosyl esters decreases with

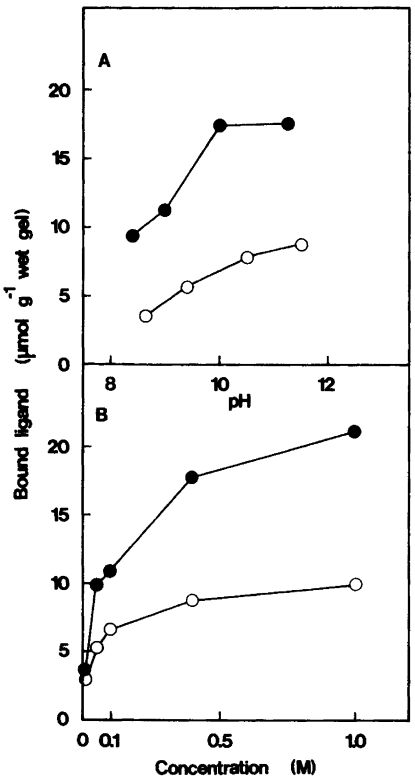

Fig. 3. Dependence on pH (A) and amount of ligand (B) during coupling of β-alanine (○) and β-mercaptopropionic acid (●) to tosyl Sepharose CL-6B (preparation C). Couplings were done for 20 h at 40 °C as described in Experimental. In the pH dependence experiment 0.4 M ligand was used and in the concentration dependence experiment the pH was 10.6 (β-alanine) and 10.0 (β-mercaptopropionic acid). In a coupling volume of 2.5 ml 1.75 g wet gel was used. The amount of bound acid was determined by titration.

increasing pH, and when the highly substituted preparation C was treated at 40 °C for 15 h at pH 10 and 13, 12 and 40% of the tosyl groups were hydrolyzed, respectively. As already mentioned, treatment with 1 M NaOH hydrolyzes nearly all the tosylates on the gel (Fig. 2).

Coupling of small ligands to tosylated agarose. β-Alanine and β-mercaptopropionic acid were used as model substances to determine how the coupling of amines and thiols depends on pH, temperature, reaction time and reagent concentration. Preparation C in Table 1 was used as tosylated gel.

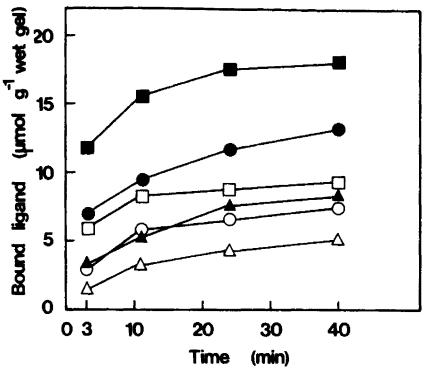

Fig. 4. Dependence of coupling time and temperature for the coupling of β-alanine (open symbols) and β-mercaptopropionic acid (closed symbols) to tosyl Sepharose CL-6B (preparation C). Three different temperatures were studied: 4 °C (△), 22 °C (○) and 40 °C (□). The ligand concentration was 0.4 M and pH 10.6 and 10.0 were used for β-alanine and β-mercaptopropionic acid, respectively. In a coupling volume of 2.5 ml 1.75 g wet gel was used. Coupling procedures are given under Experimental. The amount of bound ligand was determined by titration.

As can be seen (Figs. 3 and 4) the thiol-containing compound gave higher coupling yields throughout the experiments. The reactivity of tosylated agarose towards these nucleophiles seems to be of the same order as that reported for epoxy-activated agarose.[17] As for most coupling methods, the yield is dependent upon pH (Fig. 3 A). As expected from the pK_a values of the thiol and amine (9.5 and 10.3), it is not necessary to use higher coupling pH than 10 for the thiol, while a higher pH may be used with advantage for the amine. This is not the case for an amine with a lower pK_a, such as histamine, for which pH 10 is high enough to get a maximum yield.

Fig. 3 B shows the dependence of the degree of coupling on the concentration of the ligand. The curves obtained for the amine and thiol have the same shape and show a continuous levelling off with increasing concentration of ligand in the coupling solution. Relatively high yields of coupled ligand were obtained with low ligand concentrations in the coupling mixture.

As seen from Fig. 4, the coupling yield was higher when coupling was done at higher temperatures. A fair degree of coupling occurred within 3 h at 40 °C. The lower rate of coupling at lower temperatures could be partly compensated for by extension of the

Table 2. Coupling of 0.5 M hexylamine in dimethylformamide to tosylated agarose (preparation C in Table 1). The coupling time was 40 h. 1.75 g gel was used in a coupling volume of 2.5 ml. Coupling procedures and determination of tosyl and hexylamine are described in Experimental.

Temp. for coupling °C	Coupled hexyl-amine [a]	Remaining tosyl groups [a]	Original amount of tosyl groups [a]
40	0.32	0.80	1.15
60	0.76	0.38	1.15

[a] mmol g^{-1} dry weight preparation.

coupling time. The coupling efficiency was about the same for the amine at 20 °C as for the thiol at 4 °C.

In the highest substituted thiol preparations about 30 % of the tosyl groups were replaced by ligand (Fig. 3). The degree of displacement was much higher (Fig. 1) for the gels with lower tosyl content (about 60 %). As discussed in more detail elsewhere, it was not found necessary in our affinity studies [18] to remove all remaining tosyl groups from the gels. However, after coupling of the affinity ligand, the support can be allowed to react, if necessary with mercaptoethanol, which does not introduce any additional charges, or with ethanolamine to replace interfering tosyl groups.

Another amino compound, histamine, was also coupled to preparation C and the coupling yield was studied with respect to the same parameters as for β-alanine. The coupling behaviour was found to be roughly the same as that for β-alanine. The coupling yield with histamine was about 10–20 % higher than that with β-alanine, possibly because some additional coupling took place through imidazole nitrogen. Titration curves obtained with the histamine gels support this view.

Hexamethylenediamine, γ-aminobutyric acid and ε-aminocaproic acid were also coupled to preparation C (Table 2). The coupling yields were practically the same as those for β-alanine. Immobilized hexamethylenediamine could, in principle, act as a spacer and could be used for binding other amino compounds after treatment with glutaraldehyde.[19]

Titration of coupled products. The coupling scheme outlined in the introduction is a nucleophilic displacement by the ligand of the tosyl group on the support. Thus, a primary amino group yields a

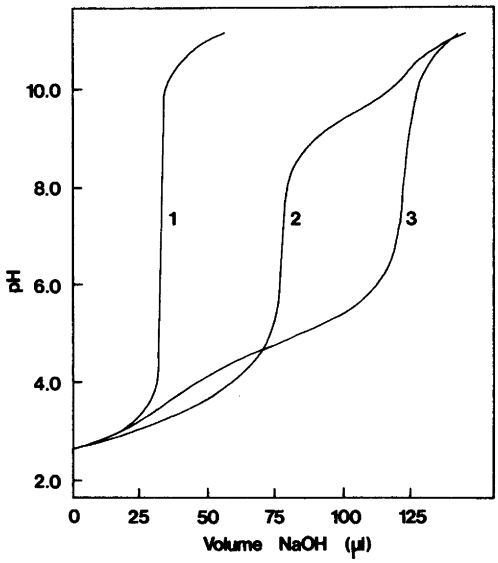

Fig. 5. Titration of (1) tosyl agarose (preparation C), (2) β-alanine agarose and (3) β-mercaptopropionic acid agarose with 0.2 M NaOH. The β-alanine and β-mercaptopropionic acid-containing gels were obtained from preparation C by coupling 0.4 M of each ligand for 24 h at 40 °C at pH 10.5 by the procedure described in Experimental. The amount of wet gel used in the titration experiment was 1.0 g.

secondary amine, a thiol group a thioether and a carboxyl group an ester when coupled to tosylated agarose. Carboxyl groups are not likely to couple to any significant extent compared with the much more reactive amino and thiol groups. In the case of β-alanine, two charged groups in equal amounts are expected to be found on the gel; in contrast the thiol compound β-mercaptopropionic acid is expected to give only one charged group on the gel. This is in accord with the titration curves obtained for these two compounds (Fig. 5). The curve for immobilized β-alanine (curve 2) indicates equal amounts of two charged groups, one with pK_a around 3.8 (carboxyl, 9 μmol g^{-1} wet gel) and one with pK_a around 9.5 (amine, 9 μmol g^{-1} wet gel). Curve 3, obtained with immobilized β-mercaptopropionic acid, indicates only one charged group with a pK_a of about 4.7 (carboxyl, 18 μmol g^{-1} wet gel).

No titrable groups could be detected in tosylated gels (curve 1) indicating that no charges were introduced on tosylation. The titration data thus indicates that a ligand can be attached by this

method without the introduction of any additional charged groups. This contrasts, for example, with the CNBr-method, which besides isoureas results in additional charged, non-characterized nitrogen-containing moieties on coupling of ligands.[10,20,21] Further, it was possible to get uncharged linkages between ligand and matrix on coupling of a thiol compound to tosylated gels. The N–C and S–C bonds most likely formed upon immobilization to tosylated agarose are expected to be extremely stable (the S–C bond is very resistant under acidic or alkaline conditions[22]). In addition, nucleophiles are not likely to cause leakage of ligands, which is in contrast with CNBr formed bonds, which also are chemically unstable.[23]

Coupling in organic solvent. Many ligands of interest in affinity chromatography are not readily soluble in water and to get high degrees of coupling of these ligands it is, therefore, advantageous if coupling can be carried out in organic solvents. To show the possibility of coupling under non-aqueous conditions, hexylamine was coupled to preparation C at two different temperatures in DMF. As is seen from Table 2 a very high degree of substitution was obtained at 60 °C with about 70 % of the tosyl groups being replaced by hexylamine. It is clear from the table that the decrease in tosyl content is almost completely "matched" by bound hexylamine. This is in agreement with the proposed coupling pathway (nucleophilic displacement) and indicates that no side reactions, such as hydrolysis or S–O bond fission, take place.

Coupling of affinity ligands and enzymes using preparation C. A number of biomolecules were coupled covalently by nucleophilic displacement to agarose preparations substituted with tosyl groups. In one series of experiments analogues of 5′-AMP and 2′-ADP were bound by their terminal amino groups to the polysaccharide support. It could be shown that from a mixture of lactate and 6-phosphogluconate dehydrogenase the immobilized AMP analogue showed bio-affinity only for NAD^+-dependent lactate dehydrogenase, whereas the immobilized ADP analogue showed affinity only for the $NADP^+$-dependent 6-phosphogluconate dehydrogenase. Furthermore, the immobilized AMP analogue (5 μmol g^{-1} wet gel) was applied for the single step purification of lactate dehydrogenase from crude beef heart extract.

To demonstrate the immobilization of proteins, soybean trypsin inhibitor was immobilized to tosylated agarose (75 mg/g dry product), tested as affinity chromatography material and shown to bind 60 mg of trypsin per g dry gel. Horse radish peroxidase and horse liver alcohol dehydrogenase were used as model enzymes. Although no optimization had been attempted, the former (approximately 70 mg/g dry support) had a coupling yield of approximately 18 % with a specific activity (relative to soluble enzyme) of approximately 10 %, whereas approximately 60 % of alcohol dehydrogenase was coupled (approximately 100 mg/g dry support) with a specific activity of approximately 25 %.

CONCLUSION

p-Toluenesulfonyl chloride has been shown to be suitable for the preparation of agarose bound ligands. Activation of the gel, leading to substitution with tosyl groups, is rapid, gives predictable levels of activation and the activated gel obtained is stable in distilled water. The swelling properties of the activated gels are practically not affected. By measuring the UV-absorbance one can easily determine the degree of substitution of the gel which is much more time-consuming, if at all possible, with most other activation methods. Water-insoluble ligands can be coupled in organic solvents. Most important, stable, uncharged, "direct" linkages between ligand and matrix are obtained without the introduction of any additional groups, thereby making it possible to devise highly specific affinity chromatographic systems. The coupling efficiency with amino or mercapto group-containing compounds compares well with other methods known.

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