Notes

A Preparation of Imidazolyl Acids Tethered to Agarose Gel and Assessment of the Degree of Coupling for Affinity Chromatography

Jaekeol Rhee

Dong-A Pharmaceutical Company Lab., Kieung-up, Kyunggido 449-900

Niels H. Andersen

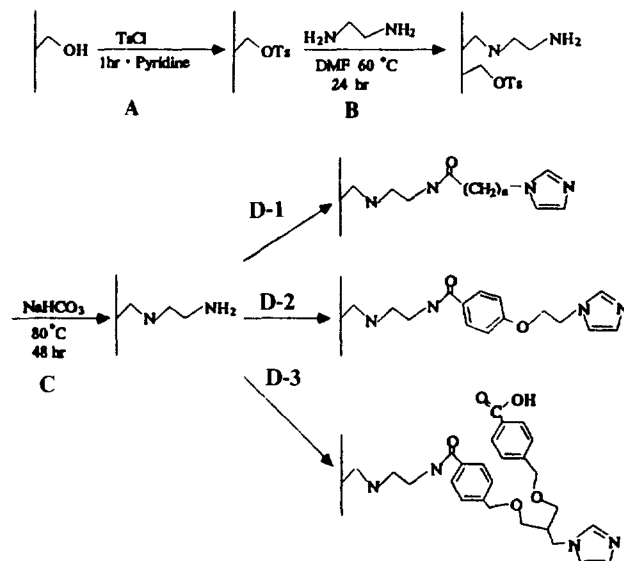
Department of Chemistry, University of Washington, Seattle, WA 98195, U.S.A.

Received January 14, 1990

Conventional procedures of protein purification generally rely on small differences in the physiochemical properties of the proteins in the mixture: solubility, charge, molecular size and shape. Isolation on the basis of these differences, by means of ion exchange chromatography, gel filtration or electrophoresis is often laborious and proceeds with modest yields. However, one of the most characteristic properties of biological macromolecules is their ability to bind other molecules in a reversible and highly specific manner. Affinity chromatography which uses this property was introduced in the 1950's[1a]; the reports by Porath and coworkers[1b-d] in which the ligand was directly attached to agarose which had been preactivated with cynogen bromide have led to a rapid and extensive development of this technique for isolation of macromolecules.

Three main components of an affinity media are matrix, spacer and ligand. The hydroxy groups on the sugar residues (for example, Sepharose as a matrix) should be first activated or derivatized for covalent attachment of a spacer or ligand. The binding site of a biological substance is often located deep within a macrocolecule, thus direct coupling of a ligand to a matrix may result in a low affinity or purification capacity of the adsorbent due to steric hindrance to complexation between the matrix to which affinity ligand is attached and other portions of the receptor-site-bearing macromolecule. To overcome this problem spacer arms are often inserted in between the matrix and the ligand to facilitate more effective exposure of ligand and to increase the capacity of the affinity adsorbent.

As a part of purifying thromboxane synthase[2d] in high yield, we needed to prepare an affinity gel which contains a ligand to bind to (interact with) the enzyme. The ligands we used in this study were inhibitors of thromboxane synthase[2], imidazolyl alkanoic acids, because they show suitable affinity for the enzyme (their IC_{50}s are 10^{-5}-10^{-7} M). In this connection, quantitative measures of gel media composition and loading are required. In addition, we report here an the easy method for measuring the amount of coupled imidazolyl acid without using radioisotope tagged species.



Scheme 1. Activation of agarose and coupling with imidazolyl acids.

Results and Discussion

Activation, Insertion of Spacer Arms. Supporting materials based on agarose have commomly been used as the matrix for affinity chromatography since agarose was introduced into affinity chromatography by Cuatrecasas and his colleagues.[3,7] In this research, we selected cross linked Sepharose 2B, which contains 2% of agarose in the gel as a supporting maxtrix, because of its high stability[4] even under drastic condition which may be required during the modification steps. Activation[12] was performed by tosylation of hydroxyl groups of agarose by following the procedures developed by Nillson[5] (see Scheme 1). The presence of tosyl groups on the tosylated gel was confirmed by UV measurement of the hydrolysate from treatment of the tosylated gel with 1 N NaOH for 60 hours at 60℃. Three characteristic peaks of tosyl group appeared on the UV spectrum at 255, 260, 266 nm (see Figure 1).

In addition to this primary activation of the gel, most modern affinity techniques require insertion of spacer arms between the activated connector (tosyl group) and the ligand (in this research, an imidazolyl acid) to provide a sterically favorable situation for the binding of proteins. In the present study, a short ethylene diamine was employed as a spacer arm, because longer arms might immobilize proteins in nonspecific manner[4], if unreacted spacer arms remained in the gel. It is also very important to remove the remaining tosyl groups (step C) for the same reasons. Thus ethylene diamine was coupled by dissolving 0.2 m*l* of ethylene diamine in DMF (0.34 m*l*) and adding 1.75 g of wet tosylated agarose, which had first been carefully freed of water by washing with DMF (700 m*l*). Coupling was done in an oil bath at 40℃ for 29 hours. After coupling the support was washed with 4×10 gel volume of water. The primary amino group

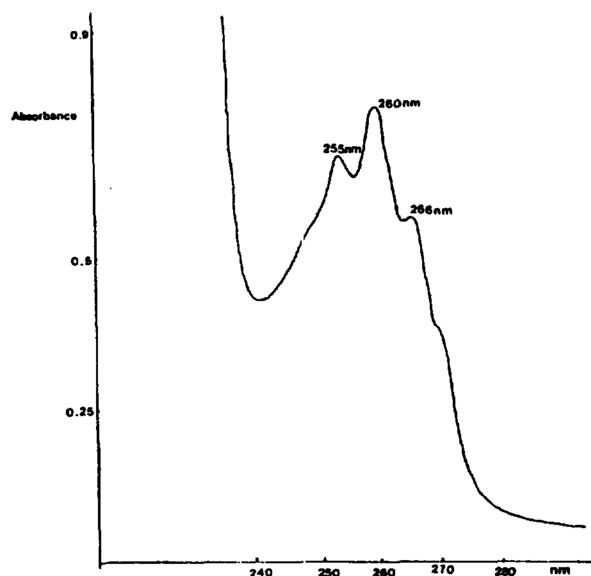


Figure 1. UV spectrum of an hydrolysate from dry tosylated sepharose 2B.

attached to the gel was identified by the TNST color test.[3a] In order to remove any unreacted tosyl groups, the above coupled gel was allowed to react with aqueous 0.5 M $NaHCO_3$ (pH 12) at 50℃ for 48 hours. The amount of amino nitrogen was also measured by a modified Kjeldahl method.[8] The yield was 0.9 mmol of tosyl group/g dry gel.

Coupling of Imidazolyl Acids to Aminoethyl-Sepharose. The condensation reaction between a COOH group and NH_2 terminal attached to Sepharose in aqueous solution was accomplished with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. An imidazolyl alkanoic acid hydrochloride (0.25 mmol) was added to 31.6 g of wet 2-aminoethyl Sepharose (0.2 mmol of amino group) in 30 m*l* of water. 1-(3-Dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (0.7 g, 3.6 mmol) dissolved in the 3 m*l* of water was added over a 65 min period to the above mixture. The pH of this suspension was brought to 4.7 with 1 N HCl aqueous solution. The reaction was carried out at room temperature for 24 hours. Coupling (for Gel G) between 2,2'[[(*p*-carboxyphenyl)methoxy]methoxy]imidazole[11] and 2-aminoethyl Sepharose 2B was carried out by pulsed addition of the carbodiimde.[6] The substituted Sepharose was then washed continuously with water until the "Lime test"[14] was negative.

Identification of an imidazolyl acid residue coupled to the gel was performed by making the corresponding imidazolyl ester after breaking the amide bond formed between COOH group of imidazolyl acid and NH_2 terminal of 2-aminoethyl Sepharose. The coupled dry gel (0.5 g) was hydrolyzed by stirring in 2 N NaOH aqueous solution (10 m*l*) at 80℃ for 24 hours and filtered. The filtrate was evaporated, acidified with 2 N HCl aqueous solution and concentrated again. The residue was heated at reflux in EtOH (or MeOH) for 4 hours. Excess EtOH was removed on a rotatory evaporator. The resulting concentrate was neutralized with saturated aqueous sodium bicarbonate solution and concentrated *in vacuo.*. The imidazolyl substituted ester formed was dissolved in methylene chloride. Retention times and mass spectra

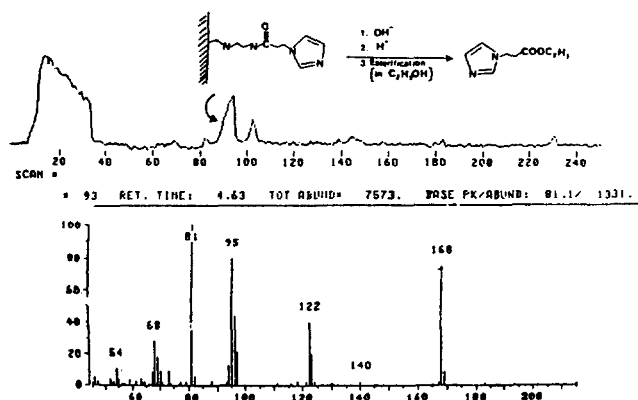
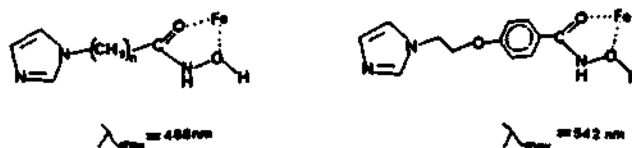


Figure 2. GC/MS of ethyl-3-(imidazol-1'-yl)-propanoate obtained from it gel-tethered form.

obtained by GC/MS (Hewlett Packard model 5985A GC/MS with a 30 mether SP-2100 fused silica capillary column) were compared with those of standard imidazolyl esters prepared independently (for example, Figure 2 shows the chromatogram of hydrolysate of Gel B and the mass spectrum of ethyl-3-(imidazol-1'-yl)-propanoate).

The degree of coupling of an imidazolyl acid residue coupled to the gel was assessed by measuring the amount of free carboxyl units COOH present in the hydrolysate of the gel. A number of methods for determining COOH units have appeared in the literature.[9,10] In this research, we followed the modified Pesez method[9], which measures the absorbance of the complex between iron (III) and a hydroxamic acid which is formed from COOH and NH_2OH. But in application of the Pesez method to the imidazolyl acids, we have found that unprotonated imidazole ring, by itself, forms a complex with $FeCl_3$, which has an strong absorbance at 400 nm. This wavelength is too close to the absorbance position (475 nm) of the anticipated complexes between iron (III) and a hydroxamic acid to give reliable results. In order to avoid this problem imidazolyl acid were used as their hydrochloride salts. Thus the hydrolysate was acidifed to pH 1 with 1 N HCl and dried at high vacuum. the resulting imidazolyl acid hydrochloride containing material was mixed with 0.1 m*l* of 2% NH_2OH in EtOH and 0.15 m*l* of 2% of dicyclohexylcarbodiimide in EtOH. The mixture was incubated at 23℃ for 1 hour and diluted to 2 m*l* by adding EtOH. The absorbance was measured at 488 nm for imidazolyl alkanoic acids and 540 nm of imidazolyl substituted benzoic acid. The amounts of acids bound to the gel were calculated from calibration curves obtained using standard imidazolyl acids. The results



are summarized in Table 1. The coupling yields (based on the content of NH_2 group in the aminoethyl gel) were found to be 16-26% depending on the hydrocarbon chain length of the imidazolyl acid. In conclusion, a variety of imidazolyl alkanoic acid tethered to CL-Sepharose 2B were prepared

Table 1. Imidazole Loadings of Gels Prepared

Gel	Ligand	Amount of ligand bound (mmol/g dry gel)
A	N~N-C(=O)-C_6H_4-O~N(imidazole)	0.17
B	N~N~N-C(=O)-$(CH_2)_2$-N(imidazole)	0.24
C	N~N~N-C(=O)-$(CH_2)_4$-N(imidazole)	0.20
D	N~N~N-C(=O)-$(CH_2)_5$-N(imidazole)	0.19
E	N~N~N-C(=O)-$(CH_2)_{10}$-N(imidazole)	0.15
F	N~N~NH_2	0.9
G	N~N~N-C(=O)-C_6H_4-O~(O~C_6H_4-COOH)~N(imidazole)	0.35

and the coupled gels were fully characterized without using any radioisotope labelled moiety.

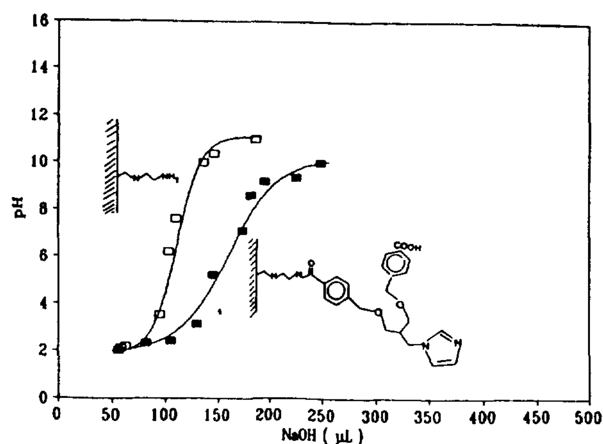


Figure 3. Titration of Gel G with 0.2 N NaOH.

References

1. (a) D. H. Campbell, Immunological Adsorbents. *Proc. Natl. Acad. Sci. U.S.A.*, **37**, 575 (1951); (b) R. Axen and J. Porath, *Nature (London)*, **214**, 1302 (1967); (c) J. Porath and R. Axen, *Nature (London)*, **215**, 1491 (1967); (d) R. Axen and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).
2. (a) S. Hammarstrom and U. Diczfalusy, *Adv. Prostaglandins and Thromboxane Research* **6**, 267 (1980); (b) K. Kato, S. Ohkwa, and S. Terao, *J. Med. Chem.*, **28**, 287 (1985); (c) For summary of thromboxane synthase inhibitors: see Ph. D. Thesis Rhee, JK: University of Washington (1989); (d) Thromboxane Synthase is an enzyme involved in transformaion of Prostaglandin H_2 to Thromboxane A_2.
3. (a) P. Cuatrescasas, *J. Biol. Chem.*, **245**, 3059 (1970); (b) P. Cuatrescasas, M. Wildkek, and C. Affinsen, *Proc. Natl. Sci. U.S.A.*, **61**, 636 (1968).
4. P. Mohr, and K. Pommerning, Affnity Chromatography, A series of monographs volume 33 (1985).
5. (a) K. Nillson, O. Norrlow, and K. Mosbach, *Acta. Chem. Scand. B*, **35**, 19 (1981); (b) K. Nillson and K. Mosbach, *Eur. J. Biochem.*, **112**, 397 (1980).
6. Sara Selfe, Ph. D. Thesis, University of Washington (1983).
7. L. Sundberg and J. Porath, *J. Chromatography*, **90**, 87 (1974).
8. J. Kohn and M. Wilchek, *Anal. Biochem.*, **115**, 375 (1981).
9. M. Pesez and J. Bartos, *Talanta*, **21**, 1306 (1974).
10. H. Miwa, M. Yamamoto, and Momose, *J. Chem. Pharm. Bull.*, **28**(2), 599 (1980).
11. For the synthesis of 2,2'[[(p-carboxyphenyl)methoxy] methyl]midazole see 2c.
12. For other activation procedures, see reference 4.
13. An attempt to identify and quantitate free acid (Ar-COOH) present in Gel G was made by titrating the gel with NaOH. Thus the gel to be analyzed was washed with 1 N KCl on a glass frit funnel. To 20 mg of dry Gel G placed in a titration vessel. 0.1 m*l* of 1 M 20 mg of dry Gel G placed in a titration vessel, 0.1 m*l* of 1 M KCl and appropriate volume of 1 N HCl was added to the desird titration volume (15 m*l*) and starting pH (2.0). Titration was carried out from pH 2.0 to 11.5 with 0.2 N NaOH recording the volume /pH dependence (as in Figure 3). However It turned out to be almost impossible to distinguish COOH (pK_a=4.7 from imidazolium ion (pK_a=6.0-6.3) in the titration curve because their pK_a's are too close to show a distinct plateau. It was also difficult to find the second equivalent pont (for titration of imidazoliun ion) clearly. But Figure 3 undisputedly indicates the presence of COOH (and /or imidazolium ion) in the gel Gel G. We can thus approximate the content of COOH assuming that the first equivalent point (for the titratioin of free COOH) may be close to the middle of the titration curve between pH 4.7 and 6.2. This suggests that the degree of coupling is 0.35 mmole/g dry gel, which is relatively high, compared with those (0.15-0.24 mmol/g dry gel) of other gels. This value is clearly subject to a greater error than the others due to the interference by the imidazolium ion and possible contaminants, and should thus be viewed as only a semi-quantitative estimate.
14. Lime test is one of methods to detect nitrogen containing compound. The procedure is as follows. A concentrated sample (<1 m*l*) was transferred into a test tube containing CaO and MnO_2 (0.1 g each). A filter paper soalked in a solution, prepared by mixing $Mn(NO_3)_2$ and $AgNO_3$ (3.5 g) in H_2O (100 m*l*) was loosely plugged onto the test tube. The test tube was heated in a flame until it became dull red. Appearance of black color on the filter paper was positive result.