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Synthesis and Characterization of an Acrylamide-based Water-soluble Affinity Polymer for Trypsin Purification

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Abstract: An acrylic polymer capable of specifically binding the enzyme trypsin has been synthesized by the copolymerization of acrylamide and *N*-acryloyl-*m*-aminobenzamidine. The object of the study was to discover suitable conditions for the synthesis of an affinity polymer which could be used in an ultrafiltration system for the biospecific affinity isolation of trypsin from mixtures containing other proteins. The polymer was purified by washing in an ultrafiltration system using a membrane with nominal molecular cutoff of 300 000. The molecular weight of the polymer product was varied by varying the concentration of initiator. The products were studied by nuclear magnetic resonance spectroscopy, by infrared spectroscopy, by viscometry and by their ability to inhibit trypsin in the absence and presence of ammonium sulphate at concentrations commonly found in crude protein solutions. No inhibition towards chymotrypsin activity was detected.

Key words: acrylamide polymer, affinity ultrafiltration, benzamidine polyacrylamide, biospecific affinity polymer, molecular recognition, trypsin, *N*-acryloyl-*m*-aminobenzamidine

INTRODUCTION

Affinity chromatography has been widely employed by biochemists and biotechnologists for over two decades as a versatile and specific approach to the purification of biological molecules. The technique, more appropriately termed 'biospecific affinity chromatography', derives its specificity and selectivity from the highly specific molecular recognition processes, which are the basis of all biological phenomena. Procedures have been designed for the purification of a very large number of biomolecules on the basis of biospecific affinity, and the application of such techniques in industry is increasing. While affinity methods have traditionally been used in downstream processing after preliminary purification steps, there is increasing interest in introducing such steps earlier in the processing procedures.

Affinity chromatography procedures are most commonly carried out in a solid phase mode. An insoluble polymer is modified by the attachment of an affinity ligand and used in a column or batch mode for the selective adsorption of the biomolecule of interest. One of the main disadvantages of using such procedures in the early stages of downstream processing is the possibility of fouling of the affinity medium by particulates in the process stream.

Another way of utilizing biospecific adsorption is the combination of cross-flow filtration techniques (i.e. ultrafiltration and microfiltration) with soluble macromolecular supports, or microparticles, carrying affinity ligands. This is a promising approach and could lead to methods amenable to use on a large scale. A few systems based on affinity ultrafiltration have been reported in the literature.[1,2] One such system was reported by Luong *et al.*,[2] who used an acrylic polymer bearing benzamidine side chains to specifically bind the proteolytic enzyme trypsin from a 1 : 1 (w/w) mixture of trypsin and chymotrypsin and retain it in an ultrafiltration device in a purification procedure, with a reported yield and purity of 90% and 98%, respectively. The utilization of a derived acrylic polymer together with filtration had previously been introduced in a method

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designed for removal of metal ions from aqueous solutions.[3,4]

Several systems utilizing microfiltration together with affinity microparticles of various types have been reported. Heat-killed yeast cells (*Saccharomyces cerevisiae*) have been used as affinity microparticles within a hollow fibre system for the purification of concanavalin A from a crude extract of Jack beans (*Canavalia ensiformis*), with 70% yields and a homogeneous product.[5] Other materials utilized as affinity microparticles include dextran granules,[6] agarose granules,[7–9] starch granules[10] and coated silica particles.[11] Finally, it should be mentioned that the methods of affinity partition[12–14] and affinity precipitation[15] rely on affinity polymers.

One advantage offered by a soluble support, at least in theory, is that the binding of protein to ligand occurs in a homogeneous phase. Whether this advantage is of any practical importance, when compared with classic solid-phase resin-attached ligand systems as in affinity column chromatography, is a question which can only be answered by (a) studying the physicochemical properties of the particular polymer used and (b) by investigating the limiting factors affecting the affinity purification system performance.

In an affinity ultrafiltration system, the affinity polymer solution is contained in a vessel from which it is recirculated through an ultrafiltration system based on membranes, or hollow fibres. Solvent lost through filtration is continuously replenished by topping up the vessel with fresh solvent. Any molecules which enter the vessel are filtered out, unless they are specifically retained by binding to the polymer, or retained by the ultrafilter due to their size. Upon binding, any unbound molecules are washed away with the ultrafiltrate, whereupon the retained molecule is specifically released by a competing solute and leaves in the filtrate. In such a system, it is important that the molecular weight cut-off of the ultrafilter be large in order to minimize dilution of the species of interest and facilitate passage through the membrane, while retaining the affinity polymer.

In this work we have investigated the conditions of synthesis of an acrylamide polymer bearing benzamidine side chains in order to produce a soluble polymer of very high molecular weight suitable for an efficient affinity ultrafiltration system. We have also characterized the polymer by physicochemical techniques and looked at the ability of the polymer to bind trypsin in aqueous solution.

EXPERIMENTAL

Materials

Acrylamide (AAm, ≥99·9%), *m*-aminobenzamidine, TEMED, urea, α-chymotrypsin (type II, bovine), trypsin (type III, bovine), N^{α}-benzoyl-DL-arginine-*p*-nitroanilide HCl (DL-BAPNA), N^{α}-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA) and succinyl-phenyl-alanine-*p*-nitroaniline (SUPPNA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Acryloyl chloride, ^2H_2O, 99·96% purity, and dimethylsulphoxide (DMSO) were purchased from Aldrich Chemical Co. Ltd (England). Ammonium peroxodisulphate was obtained from Merck (Germany) and benzamidine-HCl was obtained from Fluka Chemie AG (Switzerland).

Preparation of polymers

N-acryloyl-*m*-aminobenzamidine (N-A-*m*-AB) was synthesized by the reaction of acryloyl chloride and *m*-aminobenzamidine as described by Luong *et al.*,[2] with a yield of 40%.

The polymer synthesis was performed in batches of 1 litre reaction volumes. The total amount of monomer for each batch was 8 g, keeping a weight ratio of 1 : 5 between N-A-*m*-AB and acrylamide. N-A-*m*-AB (1·333 g) was dissolved in 10 ml of DMSO and the solution added to 800 ml aqueous solution of 6·667 g acrylamide. The mixture was filtered through a 0·22 μm filter. After extensive flushing of the solution with nitrogen gas for removal of oxygen, 20 μl of TEMED were added. The copolymerization was initiated by addition of freshly prepared ammonium persulphate solution, also nitrogen flushed. Polymers of different average molecular weight were prepared by varying the amount of initiator added. Different amounts of ammonium persulphate stock solution of concentration 5 g l^{-1}, were diluted to 190 ml prior to addition. The resulting molar ratio of ammonium persulphate to the monomers used ranged from 1 : 4100 to 1 : 75.

The reaction was allowed to proceed for 8 h, with continuous flushing by nitrogen gas. After polymerization, a 0·5 l portion of the polymer solution was transferred to a Millipore Minitan ultrafiltration system fitted with a membrane with a molecular weight (MW) cut-off of 300 000. Polymer solutions were washed with 10 volumes of urea (8 M) and then with 10 volumes of distilled water. All solutions used for washing were filtered through a 0·22 μm filter prior to use. Unless otherwise noted, analytical work was performed using the polymer retentate.

Estimation of polymer yield of molecular mass ≥300 000

Samples of the retentate (5 × 2 ml) and samples of unfiltered reaction solution were evaporated to dryness *in vacuo* for 48 h and weighed in order to estimate the amount of polymer that was retained by the membrane (yield). The estimation of polymer concentration as percentage (w/w) was carried out by the same method whenever polymer concentration needed to be con-

firmed. For this purpose, water content (percentage w/w) of dry polymer was calculated from the dry weight of the unfiltered reaction solution and assumed to be the same for the retentate.

Characterization of the copolymers

Elemental analysis was carried out using Carlo Erba Elemental Analyser Model 1106, from Strumentazione SPA (Milan, Italy). A Perkin-Elmer 1310 IR spectrometer was used to record the IR spectrum of the retained copolymer. ^1H nuclear magnetic resonance (NMR) measurement was carried out using a Bruker Amx 600 MHz spectrometer. Prior to ^1H NMR analysis, the sample taken from the polymer retentate was lyophilized and redissolved in ^2H_2O (99·96%), followed by a 2 h incubation at 80°C. The procedure of lyophilization and redissolving in ^2H_2O, followed by incubation at elevated temperature, was repeated twice. Measurements of the dynamic viscosities of polymer solutions were carried out by a falling ball viscometer according to Höppler, a product of VEB MLW (Freital, Germany). All viscosity measurements were performed at 25 ± 0·5°C, using water as a solvent. Samples were prepared from a serial dilution of concentrated retentates of washed polymer solutions. All measurements were repeated five times. Intrinsic viscosity was calculated using the relationship described in eqn (1), by least squares linear fitting and extrapolation of the experimental data:

$$[\eta] \equiv \lim_{c \to 0} \frac{\eta_{sp}}{c} \tag{1}$$

The viscosity-average molecular weight ($\bar{M}_v$) of copolymers was determined by substituting the values of $[\eta]$ in the Mark–Houwink–Sakurada equation:

$$[\eta] = KM^a \tag{2}$$

The values used for the empirical parameters, a and K, are those obtained by Scholtan:[16] 0·80 and $6{\cdot}31 \times 10^{-3}$ ($cm^3\,g^{-1}$) respectively, for PAAm at 25°C.

Microplate assay for enzyme activity and copolymer solution inhibition

Assays of enzyme activity of trypsin and chymotrypsin and the ability of copolymer solutions to inhibit enzyme activity were carried out spectrophotometrically in microtitre trays, using a ThermoMax Microplate Reader (Molecular Device Corp., Menlo Park, CA, USA). Instrument control and data analysis were effected by means of a Macintosh computer using the SOFTMax software (Molecular Devices). Basic assay conditions for trypsin activity and chymotrypsin activity were as described by Bergmeyer[17] with L-BAPNA as the substrate for trypsin and SUPPNA for chymotrypsin. Our modification of these assays allows the use of the Microplate Reader for measuring enzyme inhibition by the copolymer solutions. In short, the assay conditions were: wavelength 405 nm; final reaction volume 250 µl; light path 0·7025 cm; temperature 25°C; concentration of substrate in assay mixture 0·8 mM in the case of L-BAPNA or 4 mM in the case of SUPPNA; (extinction coefficient of the *p*-nitroaniline product ($\varepsilon^{405\,nm}$) 10 200 $M^{-1}\,cm^{-1}$);[17] buffer Tris-HCl 20 mM, pH 7·8; $CaCl_2$ 20 mM; DMSO 50 mM (trypsin assay only); 10 µl of enzyme solution were mixed with 240 µl buffer/substrate or buffer/substrate/inhibitor mixture; each measurement was performed in triplicate (mean values presented, coefficient of variation (CV) < 3% in all cases); blank reactions were measured with water replacing the enzyme solution; reactions were monitored for 3–10 min with a read interval of 10 s. All measurements of trypsin inhibition by polymer solutions and non-inhibited reference reactions were performed with 5 µg trypsin, which corresponds to 77 mU (L-BAPNA) of enzyme per well. For the chymotrypsin assay the enzyme concentration was adjusted to keep the rate of reaction below 0·03 OD/10 min.

Stock solutions of enzymes were prepared by dissolving either 10 mg trypsin or 10 mg chymotrypsin in 10 ml of ice-cold 0·0025 M HCl. Dilutions were made with ice-cold 0·0025 M HCl and the solutions kept on ice until used.

Enzyme inhibition kinetics

The N-acryloyl-*m*-aminobenzamidine–acrylamide copolymer and the free benzamidine have been shown to act as competitive inhibitors of trypsin, in respect to the substrate L-BAPNA.[2,18] The velocity of the enzyme-catalysed reaction, v, can be expressed by the well-known Michaelis–Menten equation, which in the presence of a competitive inhibitor takes the form:

$$v = \frac{[S]V}{K_M + [S] + (K_M/K_I)[I]} \tag{3}$$

where $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, V is the maximum rate of reaction and K_M and K_I are the Michaelis–Menten and inhibition constants, respectively. Equation (3) can be rearranged to the inverse form:

$$\frac{1}{v} = \frac{K_M + [S]}{[S]V} + \frac{K_M}{K_I[S]V}[I] \tag{4}$$

Now eqn (4) indicates the linear relationship of $1/v$ and $[I]$. When plotted at different substrate concentrations (the Dixon plot), a series of straight lines will be obtained, where the X and Y coordinates of the intersection point are $-K_I$ and $1/v$.

Unfortunately, this method cannot be applied for polymer solutions, as the polymer concentration, and

TABLE 1. Reactants and product of the synthesis of *N*-acryloyl-*m*-aminobenzamidine–polyacrylamide copolymer

Compound	MW	Weight used (g)	Moles used (mmol)	Ratio		Other data
				Theory	Used	
Acrylamide	70·1	6·667	93·8	27	27	
N-A-*m*-AB[a]	388·1	1·333	3·43	1	1	Complex with HNO_3
TEMED	116·2		0·13	0·04	0·04	20 μl of 6·6 M solution
$(NH_4)_2S_2O_8$	228·2	0·296	1·30	variable	0·38	
N-A-*m*-AB/PAAm copolymer[a]	$\geq 3 \times 10^5$	4·48				Yield ≈ 56% by wt, $\bar{M}_v \approx 4{\cdot}2 \times 10^5$ Monomer ratio (N-A-*m*-AB : AAm)[a] is 1 : 17 according to ^{1}H NMR analysis

[a] N-A-*m*-AB = *N*-acryloyl-*m*-aminobenzamidine; the molecular weight used here accounts for a complex with the composition of two molecules of HNO_3 per molecule of N-A-*m*-AB; AAm = acrylamide; PAAm = polyacrylamide.

therefore the concentration of inhibitor groups, cannot be detected to the required accuracy. To solve this problem, inhibition abilities of the polymer solutions were estimated by a relative inhibition:

$$\text{Relative inhibition} = 1 - \left(\frac{v_{+\text{ inhibitor}}}{v_{-\text{ inhibitor}}}\right) \quad (5)$$

where $v_{+\text{ inhibitor}}$ and $v_{-\text{ inhibitor}}$ stand for the velocity of the enzyme-catalysed reaction in the presence and absence of inhibitor, respectively. Complete inhibition gives a value of 1 and no inhibition gives a value of zero. The trypsin amount in all measurements is the same, i.e. 5 μg per well (77 mU). The rate of the non-inhibited reaction was measured to be 88 ± 1 mOD min^{-1} under the conditions of the assay.

RESULTS

Table 1 summarizes information on reactants and on the product obtained for a polymer synthesis using a monomer : initiator molar ratio of 75 : 1 in the polymerization reaction. The elemental analysis and the spectroscopic analysis were performed on this batch, using the retentate from the ultrafiltration. The results of the elemental analysis are shown in Table 2, which also includes theoretical values, calculated on the basis of different quantities of polymer-bound water. The ^{1}H NMR spectrum, together with integral values, is presented in Fig. 1.

Experimental results on the measurement of the specific viscosity of solutions of this polymer (monomer versus initiator in polymerization = 75 : 1), as well as on the specific viscosity of the largest copolymer synthesized (monomer versus initiator in polymerization = 4100 : 1), are plotted as a function of concentration in Figs 2 and 3, respectively. Values of intrinsic viscosity were obtained by least squares linear fitting and extrapolation and then used for calculating the values of $\bar{M}_v$. Results, including confidence limits (95%), are listed in Table 3.

In the trypsin assay the relationship of the initial rate of reaction and trypsin concentration was linear over the dA/dt range from 0 to 0·3 OD min^{-1} (initial rate: OD measured at 10 s intervals for 3 min), with a correlation coefficient of 0·999 for the linear regression obtained (data not shown). This detection method is linear over a wider range of dA/dt than that given by Bergmeyer.[17] The reliability of the modified trypsin

TABLE 2. Elemental analysis for N-A-*m*-AB–polyacrylamide copolymer

Element	Measured values (% w/w)	Calculated values (% w/w) for the monomer ratio 17 : 1 (AAm : N-A-*m*-AB), with the given no. of bound H_2O molecules per monomer unit:		
		0	0·5	1
C	46·76–47·10	52·42	46·97	42·55
H	6·60–6·64	6·92	7·37	7·73
N	17·17–17·28	20·05	17·96	16·27
O	28·98–39·47[a]	20·61	27·70	33·45

[a] Values given for O are estimated as the difference (in % w/w) between the sample weight and the sum of measured values obtained for the elements C, H and N.

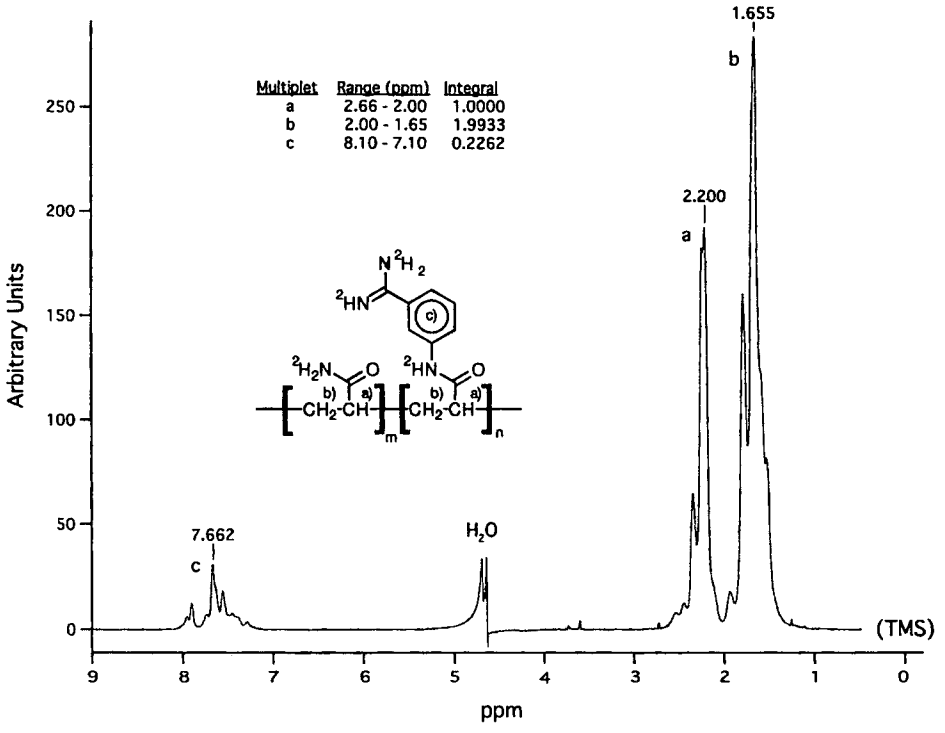

Fig. 1. ^{1}H NMR spectrum of *N*-acryloyl-*m*-aminobenzamidine–polyacrylamide copolymer. Solvent ^2H_2O. Temperature 36°C. According to the integral values, the observed monomer ratio (*m* : *n*) is 17 : 1.

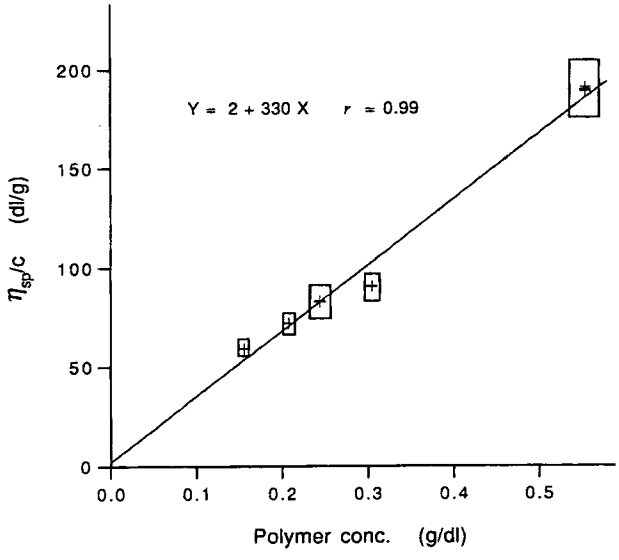

Fig. 2. Estimation of the intrinsic viscosity [η] by least squares linear regression and extrapolation for polymer product, where the ratio of monomer to initiator in synthesis was 75 : 1. Crosses (+) represent values calculated from measurements of dynamic viscosity and concentration. Squares represent the ± standard deviation of five measurements, for each of the concentration values. For each group of points, the average value for the concentration is used. According to the least squares fit the intrinsic viscosity is 2.

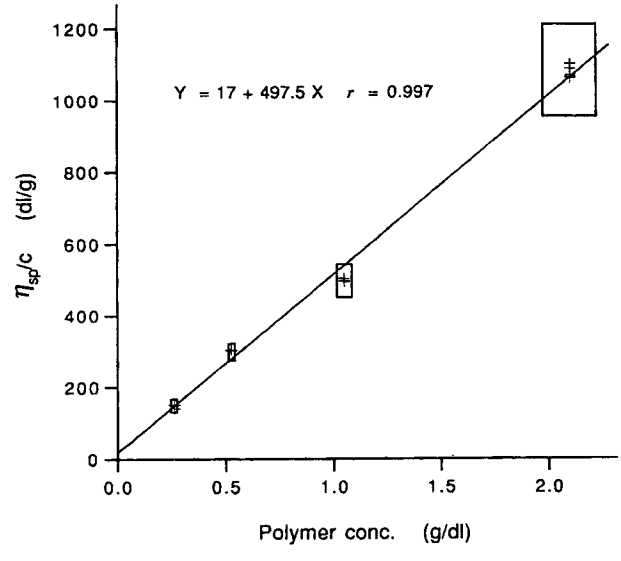

Fig. 3. Estimation of the intrinsic viscosity [η] by least squares linear fitting and extrapolation for a polymer product, where the ratio of monomer to initiator in synthesis is 4100 : 1. Crosses (+) represent values calculated from measurements of dynamic viscosity and concentration. Squares represent the ± standard deviation of five measurements, for each of the concentration values. For each group of points, the average value for the concentration is used. According to the least squares fit the intrinsic viscosity is 17.

TABLE 3. Estimation of the average molecular weight of two *N*-acryloyl-*m*-aminobenzamidine–polyacrylamide copolymers, from viscometry data

Molar ratio of monomer and initiator in synthesis	75 : 1	4100 : 1
Number of data points, n	25	20
Regression coefficient, r	0·990	0·997
Extrapolated y value at $x = 0$, a	2	17
Std dev. of a, S_a	3	10
$[\eta] \pm$ std error, $t_{.05}$	$2 \pm 1·2$	$17 \pm 4·7$
$\bar{M}_v$ and 95% confidence limits		
upper	$7·61 \times 10^5$	$8·33 \times 10^6$
mean	$4·23 \times 10^5$	$6·14 \times 10^6$
lower	$1·35 \times 10^5$	$4·10 \times 10^6$

assay was confirmed by studying the competitive inhibition kinetics of trypsin by benzamidine and comparing the results with published values from the literature. The K_I value of benzamidine was estimated from a Dixon plot to be 24×10^{-6} M, as indicated in Fig. 4.

These results are in good agreement with the results of Luong *et al.*,[2] who performed their measurements using similar reaction conditions and obtained a K_I value of $(30 \pm 3) \times 10^{-6}$ M. The concentration of affinity groups within polymer solutions could not be determined with the accuracy needed for calculations of kinetic constants. For evaluation of the inhibition ability of the 75 : 1 (monomer versus initiator in polymerization) polymer, a solution of 0·64% (w/v) was found to give approximately the same relative inhibition as benzamidine of 50 μM concentration. The concentration of affinity groups in this polymer solution was estimated by calculation, from the 0·64 (w/v) value and from the monomer composition obtained by NMR, to be around 500 μM. This indicates an approximately tenfold decrease in the inhibition ability of the affinity group upon insertion into the polymer. The relative inhibition of the other two polymer solutions (200 : 1 and 4100 : 1) was also measured, in both cases for a polymer concentration of 0·64% (w/v), and the concentration of affinity groups was again found to be around 500 μM. The results are shown in Fig. 5A. The monomer composition is assumed to be the same for the three polymer solutions, as the same fixed monomer ratio was used in their synthesis. Figure 5A shows the effect of polymer size on the inhibition of trypsin activity. Figure 5B shows the effect of ammonium sulphate on the inhibition ability of the three polymer solutions and of free benzamidine for comparison. Conditions are the same as in Fig. 5A except for the added salt.

No inhibition of chymotrypsin activity was observed when assayed in the presence of copolymer solutions (results not shown).

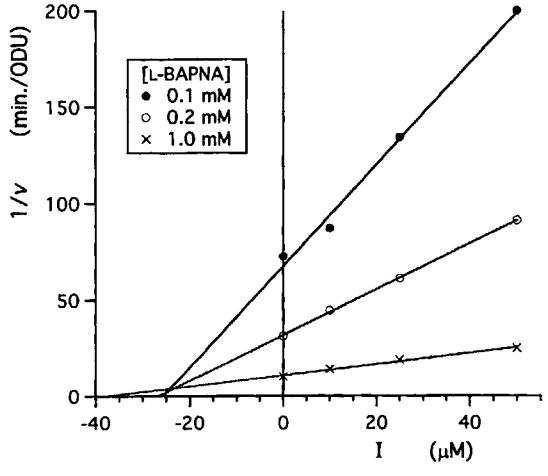

Fig. 4. Competitive inhibition kinetics of trypsin by benzamidine, represented by the Dixon plot. I = inhibitor concentration; v = velocity of reaction, presented as Δ_{ODU} (change of optical density per minute).

DISCUSSION

The chemistry of synthetic polymers has been a subject of study for more than 50 years. Nevertheless, their characterization is not necessarily straightforward or simple to perform, even for rather simple systems such as the copolymer described in this paper. Caution must be exercised in the interpretation of experimental results. This applies, among other things, to the estimation of polymer concentration and derived values such as the polymer molecular weight and composition, and the prediction of values of kinetic constants. Here we will point out some pitfalls of the methods used, as we discuss our results.

According to the NMR analysis (Fig. 1) the copolymer product has the monomer ratio of 17 : 1 (AAm : N-A-*m*-AB), which can be accepted as a reliable value. This ratio would not necessarily be expected from the

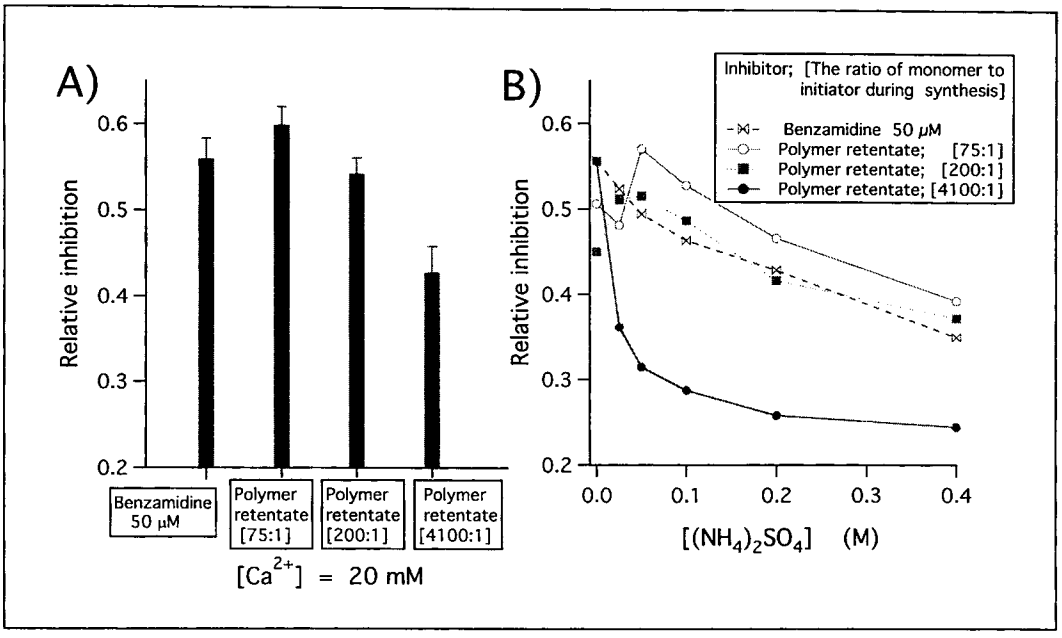

Fig. 5. (A) The effect of polymer molecular weight and (B) the effect of ammonium sulphate concentration on the ability of *N*-acryloyl-*m*- aminobenzamidine–polyacrylamide copolymer to inhibit trypsin activity. Inhibition is given in relative units compared to a non-inhibited reaction. Assay conditions: Tris-HCl 20 mM, pH 7·8; $CaCl_2$ 20 mM; L-BAPNA 0·8 mM; concentration of inhibitor, benzamidine 50 μM, or polymer 0·64% w/v (approximately equivalent to a benzamidine side group concentration of 500 μM) for the AAm versus N-A-*m*-AB ratio of 17 : 1. Bars and points show the average of five measurements with standard deviations. For each polymer the ratio of monomer to initiator from its synthesis is indicated in brackets.

initial conditions (27 : 1) of the reaction, described in Table 1.

The estimation of polymer concentration is somewhat more difficult. UV spectroscopy, which has been utilized for this purpose,[2] is of little use here, as the characteristic peaks and the total absorption spectra obtained for N-A-*m*-AB (which is an excellent chromophore) undergo a great bathochromic shift (red shift) upon incorporation in the polymer (data not shown). The same problem can arise where fluorescent groups are involved.

The elemental analysis shown in Table 2, coupled with the information on the monomer ratio in the final polymer product obtained from the NMR analysis, is consistent with the conclusion that the amount of water trapped in the crystal structure of the polymer is of the order of one water molecule per two monomer units in the polymer chain.

As described in the experimental section, the polymer concentration was estimated by weighing. The problem with the use of weighing for estimating concentration in this particular case is the hydrophilic nature of the polymer. Trapped water (i.e. water in addition to the water bound in the crystal structure) can be difficult to estimate, or to get rid of. This can lead to overestimation of the polymer concentration, which is important since both the estimation of the average molecular weight and the estimation of other values, e.g. kinetic constants such as K_I, rely heavily on an accurate estimation of the polymer concentration. One possible way around this problem, which would also give the possibility of estimating directly the concentration of affinity groups, would be to incorporate a radioactive isotope (for example ^{14}C) into the affinity monomer, prior to polymerization. Combining information from the NMR analysis with the radioactivity obtained would then give the exact concentration of the polymer solution.

The IR spectrum (not shown) of the 75 : 1 (reaction molar ratio of monomer : initiator) copolymer appeared identical to the spectrum obtained for a pure polyacrylamide polymer,[19] except for an additional sharp peak located at 2100 cm^{-1} in our spectrum, Usually peaks located in this region indicate stretching of triple bonds of alkynes (C≡C) or of nitriles (C≡N). We have not been able to correlate this peak with the functional groups expected to be found in our copolymer. The results indicate that further analysis is needed for addressing the basic structure involved.

The results of the estimation of $\bar{M}_v$ for two copolymers differing in size are presented in Table 3. The statistical procedure for estimating $[\eta]$ by linear regression requires that the X-values be measured without error. To meet this requirement, the assumption was made (Figs 2 and 3) that average values obtained for polymer concentration represented true values. For comparison, boxes indicating the standard deviations for both concentrations as specific viscosities for each point are

shown on the graphs. Table 3 shows the resulting molecular weights and the confidence limits, which are very wide despite the simplifying assumptions made. These results indicate that this popular method, widely used for the estimation of molecular weights of polymers, is not very accurate.

For the two closely related proteases used in this work, the inhibition abilities of the copolymer are limited towards trypsin activity. No inhibition towards chymotrypsin activity could be detected (data on chymotrypsin not shown). But other parameters need to be considered as well. The K_I value of free benzamidine, measured with our modified trypsin assay, lies in a range near 24×10^{-6} M, as shown in Fig. 4. Upon incorporation into the polymer, the inhibition abilities decrease by a factor of ten, as can be seen from Fig. 5. This figure also provides an indication of the behaviour of the copolymer as regards its ability to bind to trypsin, reflecting some of its physicochemical properties. Figure 5A shows the inhibition of trypsin activity by three polymers of the same concentration and composition, but differing in molecular weight. The ability of the polymer to inhibit trypsin clearly decreases with increasing polymer molecular weight. Most likely this is caused by stronger inter- and intramolecular forces of the linear polymer molecules, as they become larger. These forces may lead to coiling of the polymer backbone, resulting in the affinity groups of the copolymer becoming less available for binding because of steric hindrance. This conclusion is supported by the increase in viscosity observed as the polymer gains in molecular weight. This effect is further illustrated in Fig. 5B, where the effect of ammonium sulphate on the polymer ability to inhibit trypsin activity was measured. This experiment was performed for two reasons. (1) The sulphate ions, acting as a strong 'structure maker' on the aqueous buffer by increasing the order in the bulk water, will affect the polymer structure and inhibition ability. As expected polymers of larger molecular weight show a stronger decrease in their inhibition ability by ammonium sulphate. This is a clear example of how sensitive the polymers are to buffer composition. (2) In purification processes for trypsin or other proteins using such affinity polymers, the crude protein solutions will often contain some ammonium sulphate, which is frequently employed in preliminary purification steps. The effect of the salt on binding ability is therefore likely to be an important factor.

When planning the design of acrylamide copolymers for protein purification and for use within an ultrafiltration system, several factors have to be considered. Strong and specific binding requires optimization of the monomer ratio. For the type of copolymer described here, this work had already been done by Luong *et al.*[2] Their work and the polymer analysis described in this paper indicate that a suitable ratio is 17 : 1 (AAm : N-A-*m*-AB), for the *N*-acryloyl-*m*-aminobenzamidine–polyacrylamide copolymer. Another factor of importance, as described in this paper, is the polymer molecular weight. The efficiency of affinity ultrafiltration systems will, in general terms, be heavily affected by factors like the molecular weight cut-off value and the type of filter membrane used. The higher the cut-off value, the more efficient the filtration of the unbound proteins will be. On the other hand, increasing the polymer molecular weight will decrease the specific binding ability of the water-soluble polymer and increase the viscosity within the system. For a practical system, these factors will have to be optimized in a parallel, simultaneous manner. Finally, it should be pointed out here (data not shown) that acrylamide polymers having an average molecular weight in the range above 10^6 Da, give rise to serious viscosity problems when used within filtration systems.

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