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Affinity precipitation – an alternative to fluidized bed adsorption?

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Abstract

In affinity precipitation reversibly water-soluble affinity macroligands, i.e., polymers bearing an affinity tag, are used to first bind and than co-precipitate the target molecule. In this paper a new type of affinity macroligand is introduced. The molecules were constructed by group transfer polymerization of *N*,*N*-diethylacrylamide. They were small (less than 15 monomeric units per molecule) and very homogeneous in size (polydispersity of the molecular mass <1.2). Each base polymer started with carboxylic acid group to which site the affinity tag (the protease inhibitor *m*-aminophenylboric acid) was later bound by carbodiimid coupling (yield $>50\%$). An inhibition constant, K_I, of less than 10 μmol/l was determined for the final affinity macroligand. Low temperature and high salt concentrations were shown to be beneficial to binding. Successful affinity precipitation of the serin protease *Substilisin carlsberg* required a further lowering of the affinity constant by at least one-order of magnitude, which was accomplished by the addition of 5% (v/v) of ethylene glycol. The ethylene glycol effect was assumed to be due to the formation of a cyclic ester with the phenylboric acid of the affinity ligand.

Keywords: Affinity precipitation; Affinity macroligand; Protease

1. Introduction

The term ''affinity precipitation'' stands for two completely different approaches to specific substance isolation. One, the so-called ''primary effect affinity precipitation'', calls for at least bivalent molecules [1]. As the target molecule interacts with the added precipitation promoter, larger and larger complexes are formed, which precipitate after a certain size has been reached. The classical example of this type of affinity precipitation is the immunoprecipitation of a protein (antigen) by its antibody. A primary effect precipitation will always be a special case, the recovery of the target molecule from the precipitate can be quite difficult.

The subject of this paper is the more general ''secondary effect affinity precipitation'', where reversible soluble ''affinity macroligands'' (AMLs) are used to capture and precipitate the target substance. Below, the term affinity precipitation is used exclusively to denote such secondary effect affinity precipitation.

Affinity macroligands consist of a reversible water-soluble base polymer to which a (small) affinity mediator is linked. After the target molecule has bound to the AML via the affinity mediator, the entire affinity complex can be precipitated by changing a critical solution parameter, such as the temperature, the pH, the salt concentration, etc. [2]. Most applications favor temperature or pH/salt mediated precipitation, although precipitation by secondary cross-linking is also known [3]. From a

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biotechnological point of view, much can be said in favor of thermoprecipitation, since it requires only the input of energy but not that of matter into the system. Thus waste and sterility problems are kept to a minimum.

For thermoprecipitation from aqueous solution, the LCST (lower critical solution temperature) effect is usually exploited, i.e., precipitation is achieved by increasing the temperature. The phenomenon is not yet completely understood, however, the stability of the H-bridges between the polymer and the water molecules, which weaken with increasing temperature, and the so-called hydrophobic effect, whose influence also increases with increasing temperature, both contribute to the observed phenomenon [4]. By judicious construction of the base polymer (ratio of hydrophobic and hydrophilic/H-bridge forming groups) and the composition of the solution (salting out/salting in effects of certain salts), the LCST of the system can be adjusted to any desired point over the entire existence range of liquid water [5,6].

In many aspects the technique of affinity precipitation bears a resemblance to fluidized bed adsorption. It can be used to quickly and effectively recover the product from a complex and diluted product stream. The concentration factor can be considerable. In addition, AMLs will generally be cheaper than the stationary phase materials used in fluidized bed adsorption and the required apparatus will be considerably less complex. In spite of this, affinity precipitation has up to now failed to have a major impact on bioseparation in general and biotechnical downstream processing in particular. Some of the reasons and possible solutions will be discussed below.

In the majority of the AMLs described in the literature the base polymer is a (semi-)synthetic substance (chitosan, cellulose, poly(meth)acrylamide) and the affinity mediators are introduced either by copolymerisation with a modified comonomer or by polymer-analogous transformation of the final base polymer. In case of the synthetic polymers radical polymerization is used without exception. While such preparations have been used most successfully, some intrinsic problems are also known. As a direct consequence of the preparation, a given AML is extremely heterogeneous. While the average LCST of a homopolymer is determined by the specific and reproducible distribution of hydrophobic and hydrophilic residues along the chain (all monomer units are identical), this distribution varies considerably in a copolymer, since all copolymers tend to have a sequence length distribution. The result is a broad precipitation temperature interval and even fractionation of the AML population [7]. The affinity mediator is integrated at various positions within a given AML molecule, therefore the affinity constants also tend to vary considerably. Some authors reported a loss in the average affinity by at least one-order of magnitude once the affinity mediator had been introduced into the AMLs [8]. Last but not least, we found such molecules extremely difficult to characterize.

Some time ago we proposed a new approach to affinity precipitation using a novel type of stoichiometric polymerization for the preparation of the AML precursor. Namely the group transfer polymerization (GTP) was used to construct oligomeric (M_r<5000 g/mol) molecules of highly homogeneous structure (polydispersity M_w/M_n<1.1) and defined introduction site for the putative affinity mediator (each oligomer started with an ester group, due to the type of initiator used) [9]. It was already shown, that these base polymers have a unique and highly suitable solubility behavior and that they can be modified by hydrolysis and the coupling of, for example, an enzyme inhibitor to give a putative AML [10]. The resulting molecules were shown to have an even higher affinity to the target molecule (in terms of the inhibition constant, K_I) than the free inhibitor. However, the final AML yield after hydrolysis and coupling was rather low and the molecules were not tried for affinity precipitation at that time. In this paper we would like to report on a slightly modified synthesis protocol, which results in AML precursors bearing carboxylic acid rather than ester groups. These polymers are capable of forming covalent bonds to the affinity mediator without prior activation. The yield was much higher and the resulting AMLs were successfully used to precipitate a protease from solution.

2. Experimental

2.1. Chemicals

Chemicals were obtained from Sigma, Fluka, or

Aldrich. The highest available quality was used throughout. Unless indicated otherwise, all substances were used as obtained from the supplier. The enzyme *Subtilisin carlsberg* (crystalline, P 5380) was from Sigma. Solvents such as ethylether, tetrahydrofuran (THF), and toluene were dried by boiling over Na-wire, kept under argon atmosphere afterwards and were freshly distilled whenever needed. Dry acetonitrile was obtained from Aldrich. All other solvents were taken from the continuous distillation apparatus in the laboratory.

2.2. Spectral data

Fourier transform infra red (FT-IR) spectra were taken between 400 and 4000 cm^{-1} using a KBr pellet with 1% substance (Perkin-Elmer). The ^{1}H nuclear magnetic resonance (NMR) spectra (400 MHz) were obtained with a Bruker WM400 FT-spectrometer. Unless indicated otherwise, DMSO-d_6 was used as solvent and the temperature was 37°C. Field desorption mass spectrometry (FD-MS) was done using an Autospec-Q (VG Analytical). The sample was in solid form, a source temperature of 0°C was maintained during measurement. The positive ion mode was used. The voltage was 8 kV.

2.3. Chromatographic analysis

Reversed-phase chromatography was carried out on a Nucleosil 5 C_{18} column (Macherey-Nagel). Column dimensions were 250×4 mm. Buffer A was water (0.1% trifluoroacetic acid, TFA), buffer B acetonitrile (0.08% TFA). A 20 min linear gradient was run at 25°C from 5% B to 85% B at a flow-rate of 1 ml/min. The sample concentration was 1 mg/ml, 5 μl were injected. A detection wavelength of 214 nm was adjusted. For gel permeation chromatography a Spectra-Physics SP 8100 chromatography system equipped with a differential refractive index detector (Waters) and a UV detector (Labomatic, 230 nm) was used. Two PL-Gel 5 μm columns (50 nm, dimensions 300×7.7 mm) were used in series. The mobile phase was dry THF, the flow-rate 1 ml/min. The sample concentration was 1 mg/ml, 100 μl sample were injected. A temperature of 55°C was maintained.

2.4. LCST determination

The transmission of an aqueous solution of a given polymer as a function of the temperature was measured at 500 nm on an Uvicon 860 spectral photometer. Readings were taken for a temperature interval of 10°C around the expected precipitation temperature. Heating rates were 1°C/10 min.

2.5. Calculation of the molecular mass/the polydispersity

The apparent weight average of the molecular mass, M_w, and the apparent number average of the molecular mass, M_n, were calculated from the mass spectra using the following formulas:

$$M_w = \sum N_i M_i^2 / \sum N_i M_i \text{ and } M_n = \sum N_i M_i / \sum M_i$$

with M_i: mass of a given unimolecular oligomer species in a given sample and N_i: number of molecules of that weight in the preparation.

The degree of polydispersity was calculated as $P = M_w / M_n$.

2.6. Synthesizes

The monomer *N,N*-diethylacrylamide was prepared as described in Ref. [11]. The final product was stirred over CaH_2 for 24 h and vacuum distilled. $CuCl_2$ was added as radical inhibitor. The final purity was ascertained by gas chromatography (GC). Yields were between 50 and 60%, purities 100%. The pure and dry monomer was stored in a septum-sealed flask at −20°C.

The GTP initiator dimethylketene bistrimethylsilyl acetale was synthesized according to Ref. [12]. The yield was 66% and the purity >95%. The purity was ascertained by ^{1}H-NMR ($CDCl_3$): $\delta = 0.16$ [$Si(CH_3)_2$] and $\delta = 1.48$ (CH_3); intensity ratio 18:6; the boiling point of the substance was 68–72°C/20 mbar.

2.7. Polymerization

Glassware was heated to 300°C under argon for several hours until dry. Syringes were stored at 50°C. Glass ware, syringes, agents, and solutions were handled under a moisture-free argon atmosphere

after cleaning (purification) and drying. The base polymers were prepared by group transfer polymerization as previously published [9]. Thirty ml of dry THF were placed under argon in a dry, septum-sealed flask equipped with a magnetic stirrer. Eighteen ml monomer and 2.3 ml initiator were added next (molar ratio monomer:initiator = 15). The mixture was cooled to 0°C (ice bath). The reaction was started by the rapid injection of 250 μl of the catalyst solution (0.04 mol/l $NBu_4^+OAc^-$ in THF) to the well stirred mixture. After 4 h the reaction was stopped by adding 1 ml high purity water. Afterwards the solution was evaporated in vacuum (10^{-3} mbar) and the residue dissolved in THF. The polymer was precipitated from petroleum ether and dried in vacuum (10^{-3} mbar) until constant weight. As a result of the initiation reaction, each polymer produced by this reaction starts with a carboxylic group. Alternatively, dimethylketenemethyl trimethylsilyl acetale (used as obtained from Aldrich) was used as initiator. The Monomer:initiator ratio was 10 (12 ml monomer in 20 ml THF) and 258 μl catalyst were added. The reaction was stopped in this case by the addition of methanol instead of water. Polymers produced by this method carry an methylester end-group.

2.8. AML synthesis

The direct synthesis of the AMLs required a base polymer with a carboxylic acid end group. 1.35 g of the polymer were dissolved in 125 ml high-purity water. The pH was adjusted to 5.0 with 2 *M* NaOH. The solution was cooled to 0°C and 1.8 g EDC [*N*-ethyl-*N′*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (9.39 m*M*)] were added. The solution of 1.86 g *m*-amino-phenylboric acid hemisulfate (10 m*M*) in 25 ml high-purity water (pH of 5 adjusted with 2 *M* NaOH) was added drop-wise over a time span of 20 min. A pH of less than 5.5 was maintained by adding 2 *M* HCl if necessary. After 3 h the pH remained constant and the reaction was considered finished. The solution was nevertheless kept overnight at 0°C. After adjusting the pH to 2.0 (2 *M* HCl) the solution was maintained at 37°C for 2 h and then heated within 30 min to 50°C. The formed precipitate was isolated and redissolved in ice-cold water (150 ml). The procedure was repeated three times, then the final precipitate was lyophilized. Following this the AML was dissolved in 75 ml acetylacetic ester, precipitated from 1 l of petrol ether and dried in vacuum (10^{-3} mbar).

In the case of a base polymer bearing an ester end group, a hydrolysis was necessary. Four g of polymer were treated with enough 6 *M* NaOH to assure a molar excess of 50. After 16 h at 0°C the reaction was stopped by lowering the pH to 2.0 (5 *M* HCl). The polymer was precipitated at 50°C, the precipitate recovered by centrifugation (30 min, 10 000 *g*, 50°C), and the final product lyophilized. The content of carboxylic groups was determined by titration. A 3% (w/w) solution of the polymer was prepared in water and the mixture titrated with 0.05 *M* NaOH until a pH value of 9 was surpassed.

For coupling of the inhibitor, the hydrolysate was treated as described above for the carboxylic end group polymer save for the final precipitation from petrol ether, which was not carried out in this case. Seventy-five ml of a 4% polymer solution, 111 mg of *m*-amino-phenylboric acid hemisulfate (in 10 ml water), and a two-times excess of EDC were used.

2.9. Inhibition constants

The inhibition of *Subtilisin carlsberg* by the AML or the model inhibitor was established using the artificial substrate *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, which releases a colored substance upon proteolysis. Reaction buffers were 0.2 mol/l. For pH values between 6 and 8 sodium phosphate was used, between 8.5 and 9.5 pyrophosphate was used. A 0.125 mol/l substrate solution was prepared in the buffer (2 mmol/l, pH 6.0). The enzyme solution (1 mg/75 ml) was prepared in the same buffer. Data points were taken for 0 μl, 50 μl, 100 μl, 150 μl and 200 μl of inhibitor solution (prepared in pure water). The inhibitor solution, 100 μl reaction buffer, and 500 μl substrate solution were diluted with water to give 800 μl of total solution and mixed well. After 5 min of incubation at a given reaction temperature, 50 μl enzyme solution was added, the solution briefly mixed and the release of *p*-nitroanilide followed at 410 nm for a suitable time. If non-linearity was observed at the end of the reaction, only the initial slopes were taken. An absorption coefficient *e* of 8800 cm^{-1} ml^{-1} was found to be near constant over

the investigated pH and temperature interval. Average values for the inhibition constant K_I were calculated from the corresponding Dixon-type plots according to Bergmeyer [13].

2.10. Affinity precipitation

A 0.5% solution of the AML was prepared in pure water. The indicated amount of this solution was mixed with a certain amount of a 0.1% solution of *Subtilisin carlsberg* in 10% Na_2SO_4 of the indicated pH and an additional amount of the indicated buffer (usually 0.2 mol/l sodium phosphate). Sometimes a second salt solution was added in the hope of improving the yields. The mixture was kept at 0°C for 1 h and then heated to 25°C. Unless indicated otherwise, the precipitation was allowed to proceed for an hour, afterwards the precipitate was recovered by centrifugation (25°C, 10 000 *g*, 10 min). The reference was a similar mixture containing an equal amount of pure water instead of the AML solution. The success of the precipitation was estimated by measuring the residual activity in both the supernatant of the AML precipitate and the reference solution. A second set of affinity precipitation was carried out under similar conditions, except for the fact that a 2% AML solution was used and 50 μl glycerol was added to the respective mixtures.

3. Results and discussion

3.1. Synthesis of the affinity macroligand base polymer

The AML base polymer, poly-*N*,*N*-diethylacrylamide, was prepared by GTP. The putative reaction scheme is given in Fig. 1. In order to provide each polymer with a carboxylic acid group, dimethylketene bistrimethylsilyl acetale was used to initiate the polymerization instead of the GTP initiators used previously [9]. The overall yield of the polymer was low, i.e., only 12%. The desired structure was obtained, however, as we were able to confirm by IR [bands at 1730 cm^{-1} (free acid), 1568 cm^{-1} and 1412 cm^{-1} (sodium carboxylate ion), respectively] and ^{1}H-NMR (12.2 ppm/d_6-DMSO) spectroscopy. By titration of the end group against 0.05 *M* NaOH, an average number of 11 monomer units per polymer was calculated. The p*K* of the polymer was concomitantly determined to be 4.9.

The FD-MS spectrum of the base polymer is shown in Fig. 2. If the polymer molecules are assumed to form dimers and attach two sodium ions, the signal sequence corresponds well to a putative polymer structure of one acid end group (87.1 g/mol), a varying number of monomer units (127.1 g/mol each), and a terminating proton (1.0 g/mol). The formation of dimers is highly probable under

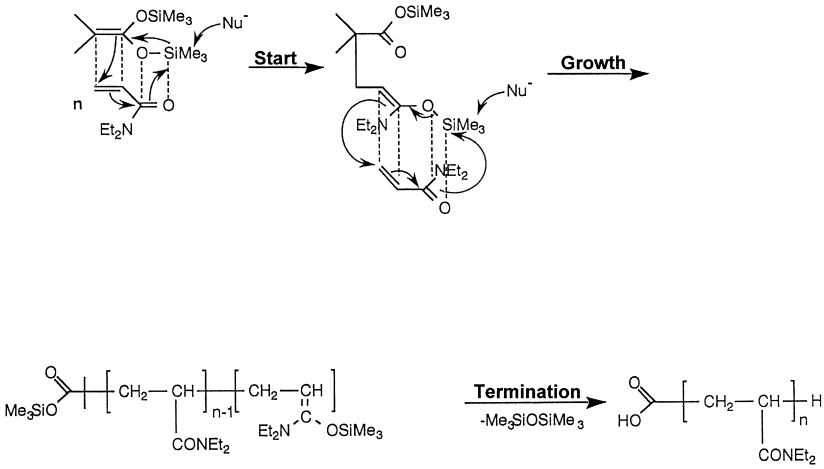

Fig. 1. Reaction mechanism presumed for the group transfer polymerization of *N*,*N*-diethylacrylamide using dimethylketene bis trimethylsilyl acetale as initiator and $NBu_4^+OAc^-$ as nucleophilic catalyst.

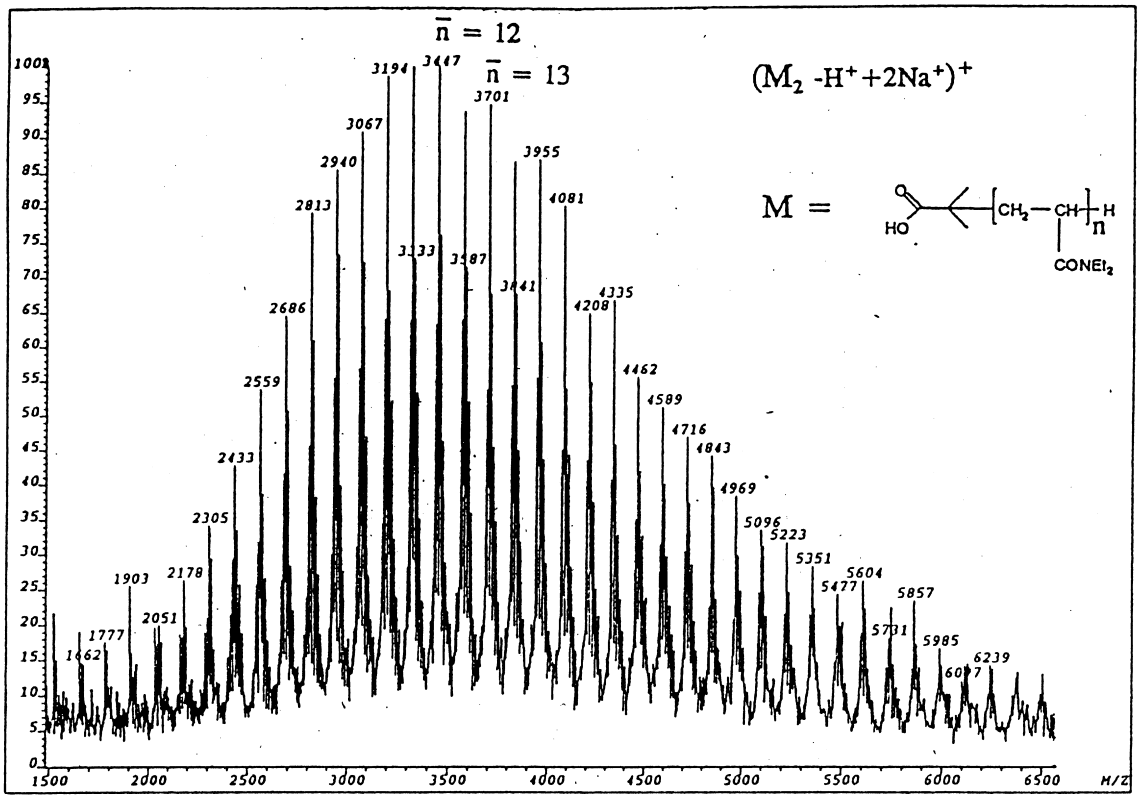

Fig. 2. Field desorption mass spectrum (FD-MS) of poly-*N,N*-diethylacrylamide with carboxylic end group used as base polymer for affinity macroligand construction. The assumed structure is shown in the insert. In the formula and the spectrum, *n* stands for the number of monomer units incorporated into the polymer (degree of polymerization).

FD-MS conditions. Again an average degree of polymerization of 11–13 can be calculated from the spectrum.

An LCST of approximately 30.5°C was observed for a 0.05% (w/w) aqueous solution of the pure polymer. This is identical to the value measured for similar GTP poly-*N,N*-diethylacrylamide structures with ester end groups [6]. Therefore, the thermo-precipitation behavior of the base polymer seems to be determined by the monomer units and not by the end group. This is further corroborated by the fact that the pH, i.e., the presence or absence of a charged structure in the molecule also has no influence on the LCST in this case, Fig. 3.

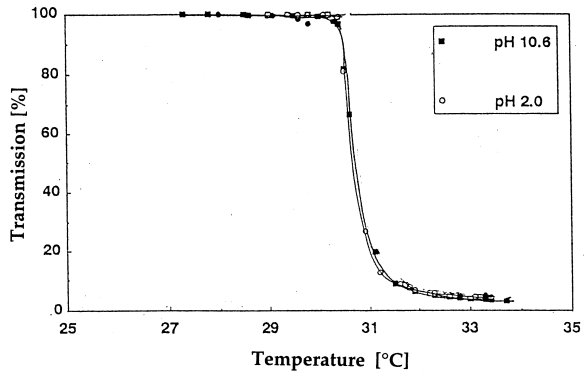

Fig. 3. Solubility of poly-*N,N*-diethylacrylamide in pure water as a function of the temperature (precipitation curve). The curve was recorded for two pH values to investigate the effect of the presence or absence of a charge in the molecular structure on the precipitation temperature. At a pH of 10.6 the carboxylic end groups should all be deprotonated.

3.2. Construction of the affinity macroligand

The serine protease *Subtilisin carlsberg* was used

as an example for a biotech product suitable for enrichment by affinity precipitation. This enzyme and related *Subtilisin* types is produced by various species of *Bacillus*, including a thermophilic *Bacillus licheniformis* [14] and has, for example, potential as a washing powder additive. The desirable degree of final purity is lower for such a technical product than for most other bioproducts, hence the simple purification. *Subtilisin* shows a certain tendency for autoproteolysis especially at higher concentrations. The choice of an inhibitor as affinity mediator introduces both a capturing and a stabilizing agent into the system. Since the affinity mediator should be small and of high affinity, the selection was limited to molecules like phenylarsenic acid [15] or phenylboric acid [16] for inhibition of the amidolytic activity of *Subtilisin*.

In the end, *m*-aminophenylboric acid was used as affinity mediator. Based on previous experience, the substitution of the aromatic ring was expected to both increase the affinity to the protease and allow a coupling to the carboxylic acid groups of the AML precursor by the carbodiimid reaction [10].

A major drawback of the AML based on ester end group polymers had been the low content of fully active molecules in the final AML preparation (<2%) [10]. When *m*-aminophenylboric acid was linked to the novel type of base polymer bearing the carboxylic acid instead, a yield of more than 50% (52.2%) of fully active AML in the final preparation was determined by titration against 0.05 *M* NaOH. The p*K* of the AML was 8.5. The structure was verified by ^{1}H-NMR, Fig. 4.

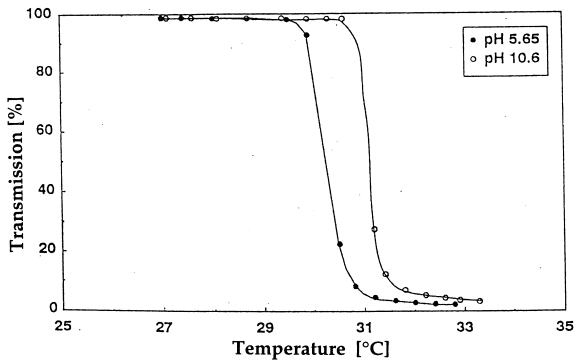

Fig. 5. Solubility of the affinity macroligand in pure water as a function of the temperature (precipitation curve). The curve was recorded for two pH values to investigate the effect of the presence or absence of a charge in the molecular structure on the precipitation temperature. At a pH of 10.6 all phenylboric acid end groups should be deprotonated.

According to reversed-phase (RPC) and gel permeation (GPC) chromatography the final AML preparation was free of non-bound inhibitor molecules and residual monomers. The solubility of the AML was similar to that of the base polymer, Fig. 5.

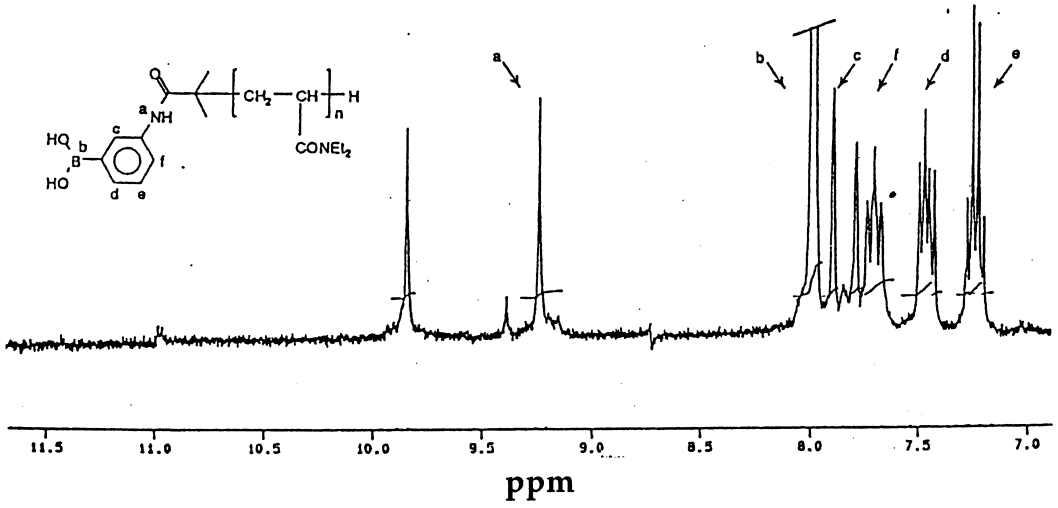

Fig. 4. High-resolution (600 MHz) ^{1}H-NMR of the synthesized affinity macroligand (d_6-DMSO, 37°C). The assumed molecular structure is indicated.

However, a slight pH dependency of the LCST was observed and the deprotonation at elevated pH (10.6) resulted in an increase of the precipitation temperature by 1°C.

3.3. Inhibitor qualities of the AML

The substrate specificity of *Subtilisin* is low [17]. The artificial substrate succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide can be used to evaluate the enzyme activity, since the *p*-nitroanilide released from this substrate upon hydrolysis is easily detected.

The Dixon plot constructed for three substrate concentrations, Fig. 6, clearly showed competitive inhibition of the enzyme by the AML. Thus the new AML showed a behavior quite similar to that observed previously for both the free inhibitor phenylboric acid and the an AML model constructed on the basis of pivalic acid [10]. All calculations of inhibition constants were based on the concentration of active AML. Non-activated polymer molecules were not considered.

A logarithmic plot of the reaction rate in the presence, v_i, and the absence, v, of the inhibitor (AML) against the inhibitor, [I], and the substrate, [S], concentration as well as the inhibition, K_I, and the Michaelis, K_M, constant according to the following formula:

$$-\log(v/v_i - 1) = -\log[I] + \log[K_I(1 + [S]/K_M)]$$

gave a slope of 1. The only explanation for this was the assumption of a stoichiometric reaction between the enzyme and the AML.

The AML showed a pronounced dependency of the inhibition constant on the pH, Fig. 7. The optimum, i.e., a K_I of ca. 15 μmol/l, was found between pH values of 7.5 and 8.0. The inhibition became less pronounced at high pH, because deprotonated phenyl boric acid is not capable of inhibition (p*K* phenylboric acid/phenyl boronate = 8.83). At very low pH, on the other hand, the concentration of active, i.e., deprotonated, *Substilisin carlsberg* is low (p*K* of the His64 in the active center ca. 6.7).

The inhibition constant could be lowered even further by reducing the temperature. A value of 4.0 μmol/l was measured at pH 7.5 and 11.2°C. The observation was especially important, because the *Substilisin* used in these experiments had a temperature optimum of the proteolytic activity of about 55°C. Performing the affinity precipitation at low temperature would concomitantly increase the binding strength of the enzyme–AML complex and reduce the tendency for autoproteolysis, which is always a problem during the isolation of proteases.

3.4. Affinity precipitation without binding enhancement

In affinity chromatography, affinity ligands with

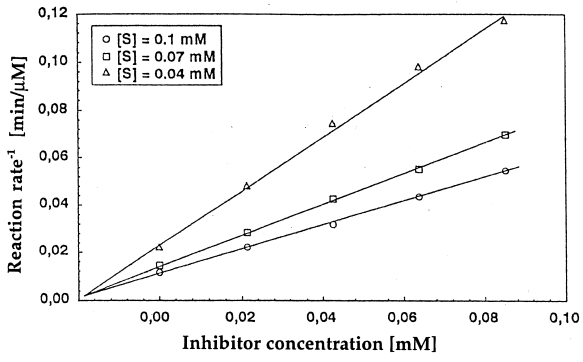

Fig. 6. Inhibition of the amidolytic activity of *Substilisin carlsberg* by the affinity macroligand (plot according to Dixon). Substrate: Succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, temperature: 25°C, buffer: 0.02 mol/l sodium phosphate pH 6.5. The intercept corresponds to an inhibition constant, K_I, of 19.5 μmol/l. The inhibition is competitive.

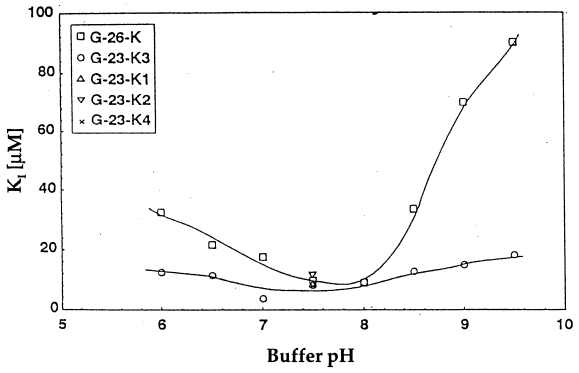

Fig. 7. Dependency of the inhibition of the amidolytic activity of *Subtilisin carlsberg* by the affinity macroligand on the pH. Substrate: Succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, temperature: 25°C, buffer: 0.02 mol/l sodium phosphate, pH 6–8 or 0.02 mol/l sodium pyrophosphate, pH 8.5–9.5.

binding constants between 10^{-4} and 10^{-8} mol/l are recommended. In the case of much higher constants the binding is most likely too weak, much lower constants will make the recovery of the product difficult. With an inhibition constant below 10^{-5} mol/l, the AML's affinity to the target molecule should in principle be sufficient for an affinity separation. Whether the fairly small AML molecules would indeed be capable of precipitating a significant amount of enzyme remained to be seen.

In the ensuing experiments, we generally found it useful to work at fairly high salt concentrations in order to reduce the autoproteolytic tendency of the enzyme. The inhibition was concomitantly enhanced, as Fig. 8a–d demonstrate for ammonium sulfate, sodium sulfate, ammonium chloride and sodium chloride. The LCST was lowered by the salt addition and affinity precipitation became possible at 25°C. Unspecific protein precipitation by the salt addition was not observed. The AML concentration in the supernatant was below the detection limit of the mass spectrometer. Previous work [18] with similar AML using quantitative gel–sol separation by temperature-controlled centrifugation also showed that more than 99% of the polymers are found afterwards in the precipitate.

The results of the first set of affinity precipitations carried out in sodium phosphate buffer for various pH at 0°C are compiled in Table 1. None of the experiments resulted in an appreciable reduction of the enzyme activity in the supernatant of the precipitate in relation to that of the reference. The addition of salts, Table 2, or a variation of the enzyme:AML ratio did not improve matters.

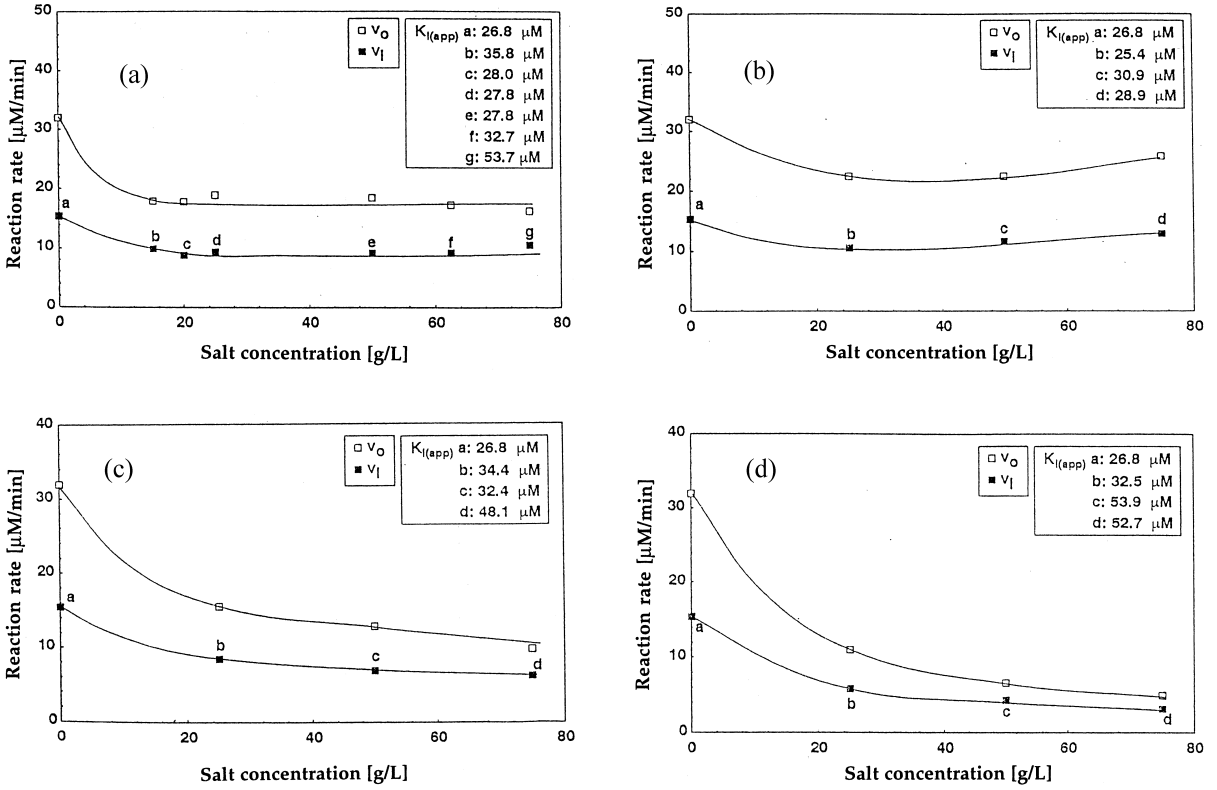

Fig. 8. Influence of salt addition on the inhibition of the amidolytic activity of *Subtilisin carlsberg* by the affinity macroligand model *N*-pivalyl-*m*-amino-phenylboric acid. Substrate: 0.1 m*M* succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, inhibitor concentration: 28.69 μmol/l, temperature: 25°C, buffer: 0.05 mol/l sodium phosphate, pH 7.5. The apparent inhibition constant K_{Iapp} was calculated by: $v_0/v_i = 1 + [I]/K_{Iapp}$ with v_0: reaction rate without inhibition, v_i: reaction rate with inhibition, [I] concentration of the inhibitor. (a) $(NH_4)_2SO_4$; (b) Na_2SO_4; (c) NaCl; (d) NH_4Cl.

Table 1
Affinity precipitation at different pH values

	pH					
	6.0	7.0	8.0	8.5[a]	9.0[a]	9.5[a]
Activity change: sample/reference (%)	+7.7	+2.7	−1.4	+3.9	−2.9	−0.2

Sample: AML solution: 500 μl 0.5% (w/w) in water, enzyme solution: 0.5 mg/ml of 10% Na_2SO_4 solution 250 μl, buffer: 250 μl 0.2 mol/l sodium phosphate ([a] 0.2 mol/l sodium pyrophosphate).
Reference: same mixture except for the addition of 500 μl of pure water instead of the AML solution.

3.5. Affinity precipitation with binding enhancement

The failure to precipitate an appreciable amount of enzyme could have had two reasons. It is possible that the AML was to small and thus not capable of coprecipitating the larger enzyme. Instead of transferring its solubility character to the protein by the affinity interaction, the protein had been transferring its solubility character to the oligomeric AML. A second possibility was that the binding between the two affinity reactants was not strong enough. Since the majority of the affinity precipitation described in the literature used badly characterized AML, little is known in regard to optimum binding constants between AML and target molecule in affinity precipitation. As pointed out above, a value of 10 μmol/l would be within the range desirable for affinity chromatography, but only just so. A higher binding strength would definitely be desirable. From publications in the area of affinity chromatography [19] it is known that the binding of *Subtilisin* to phenylboric acid ligands can be enhanced by the addition of glycerol. It is assumed that the glycerol reacts with the phenylboric acid to give an ester (five-membered ring) which in turn has a higher affinity to the enzyme, Fig. 9.

The effect of the formation of a phenylboric ester on the inhibition of *Substilisin carlsberg* was investigated using pivalyl-*m*-aminophenylboric acid as model inhibitor. The model substance mimics the chemical environment of the inhibitor ligand in the AML to a high degree and had previously been demonstrated to be a good model. Fig. 10a demonstrates that the addition of up to 25% (v/v) does

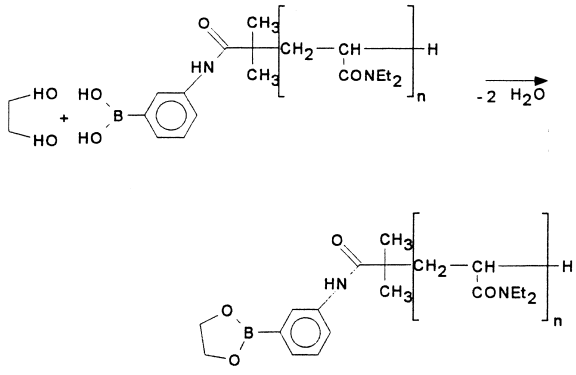

Fig. 9. Putative structure of the cyclic phenylboric acid ester.

Table 2
Affinity precipitation in the presence of different salts

	Salt concentration (%, w/w)					
	10	20	30	10[a]	20[a]	30[a]
Activity change: sample/reference (%)	+2.9	+4.9	−2.5	+6.6	+2.7	+3.2

Sample: AML solution: 500 μl 0.5% (w/w) in water, enzyme solution: 1 mg per 10 ml of water 300 μl 0.1 mg/ml of enzyme in water, buffer: 100 μl 0.2 mol/l sodium phosphate, pH 7.5, salt solution: 100 μl Na_2SO_4 [[a] $(NH_4)_2SO_4$).
Reference: same mixture except for the addition of 500 μl of pure water instead of the AML solution.

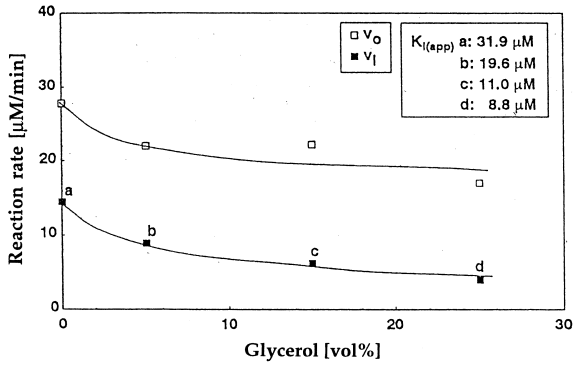

Fig. 10. (a) Influence of a glycerol addition on the inhibition of *Subtilisin carlsberg* by the affinity macroligand model *N*-pivalyl-*m*-amino-phenylboric acid. Substrate: 0.1 m*M* succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, inhibitor concentration: 28.69 μmol/l, temperature: 25°C, buffer: 0.05 mol/l sodium phosphate, pH 7.5. The apparent inhibition constant $K_{I_{app}}$ was calculated by: $v_0/v_i = 1+[I]/K_{I_{app}}$ with v_0: reaction rate without inhibition, v_i: reaction rate with inhibition, [I] concentration of the inhibitor. (b) Influence of an ethylene glycol addition on the inhibition of *Subtilisin carlsberg* by the affinity macroligand model *N*-pivalyl-*m*-amino-phenylboric acid. Conditions as in (a).

indeed increase the binding strength between the enzyme and its inhibitor. An even more pronounced effect could be produced by the addition of similar amounts of ethylene glycol, Fig. 10b. A verification of the effect for the AML showed that the addition of 5% (v/v) of ethylene glycol lowered the K_I by a factor of 4.

When an affinity precipitation was attempted in the presence of 5% ethylene glycol, for the first time the enzyme was indeed coprecipitated under the circumstances, Table 3. We observe a significant lowering of the enzyme activity in the supernatant of the precipitate compared to the reference solution. From these results it can be deduced, that a high binding strength is very important in affinity precipitation, while on the other hand oligomeric AML can be used.

The ''yield'' in the above mentioned affinity precipitations increased with increasing enzyme concentration, presumably the capacity of the AML preparation is by no means fully exploited. This was further corroborated by similar precipitation experiments carried out with an AML preparation prepared the conventional way, i.e., by hydrolysis of an ester end group to yield the carboxylic acid groups used for anchoring the affinity mediator. The amount of active AML in the entire preparation was less than 4% (3.8%) in this case. A series of affinity precipitations carried out with this AML preparation yielded nevertheless similar results in terms of yields.

By using a phenylboric ester instead of the free phenylboric acid as inhibitor, the affinity precipitation became also less pH dependent. Since no deprotonation was possible, the reaction could take place even at a pH of 8.5 or 9.0, which also improved the ''yields'' considerably, Table 4. The

Table 3
Affinity precipitation in the presence of 5% (v/v) ethylene glycol

	Enzyme solution (μl)					
	350	250	150	50	25	100[a]
Salt solution (μl)	0	100	200	300	325	0
Activity change: sample/reference (%)	−28.0	−15.4	−9.2	−10.2	+1.8	−11.6

Sample: AML solution: 500 μl 2% (w/w) in water ([a] 750 μl), enzyme solution: 0.5% mg/ml of enzyme in 10% Na_2SO_4, buffer: 100 μl 0.5 mol/l sodium phosphate, pH 7.5, salt solution: 10% Na_2SO_4, ethylene glycol: 50 μl.
Reference: same mixture except for the addition of 500 μl (750 μl) of pure water instead of the AML solution.

Table 4
Affinity precipitation using a low-activity AML preparation

pH	Salt solution (μl)	Enzyme solution (μl)	Activity change: sample/reference (%)
7.0	0	350	−19.34
7.0	100	250	−25.63
7.5	0	350	−16.75
7.5	100	250	−31.60
8.0	0	350	−19.99
8.0	100	250	−25.78
8.5	0	350	−38.27
8.5	100	250	−28.42
9.0	0	350	−39.44
9.0	100	250	−32.93

Sample: AML solution: 500 μl 1.0% (w/w) in water, enzyme solution: 0.1 mg/ml of 10% Na_2SO_4 solution, buffer: 100 μl 0.5 mol/l sodium phosphate of the indicated pH, salt solution: 10% Na_2SO_4 in water, ethylene glycol: 50 μl.
Reference: same mixture except for the addition of 500 μl of pure water instead of the AML solution.

salt addition had a pronouncedly positive effect close to neutral pH, but became less beneficial and even detrimental above a buffer pH of 8.

4. Conclusions

Affinity precipitations have a chance to become cheap and efficient alternatives to fluidized bed adsorption either for product isolation or for the removal of certain key impurities such as proteases, DNA, or even endotoxins. However, the active agent, i.e., the affinity macroligand, needs to become accessible for full characterization. Otherwise process design and scale-up becomes difficult. We have in the past worked on the construction of suitable molecules which have predictable, reproducible and controllable biological (i.e., inhibition constants) and polymer chemical (i.e., precipitation temperature) properties. That it is indeed possible to use these constructs for the precipitation of proteases has been shown in this paper.

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