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Peptidyl methyl ketones as ligands in affinity chromatography of serine and cysteine proteinases

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ABSTRACT

Peptidyl methyl ketones are reversible inhibitors of serine and cysteine proteinases and can be employed as ligands in affinity chromatography. Serine proteinases of the subtilisin family may be purified using the tripeptidyl methyl ketone $-Ala_2-PheCH_3$ as ligand, whereas for papain, the best-known cysteine proteinase, both the $-Ala_2-PheCH_3$ and the $-Phe-AlaCH_3$ ligands are efficient. Complete elution of affinity-bound proteinases with isopropanol was demonstrated by investigations with thermitase, a subtilisin-type serine proteinase, which was ^{14}C labelled. Bound proteinases with different affinity for a defined ligand may be consecutively eluted using increasing concentrations of isopropanol in the elution buffer, as was shown, for example, with a mixture of thermitase and subtilisin DY. The quality of the lyophilized affinity-purified thermitase is similar to that of thermitase preparations purified by isoelectric focusing and adsorption on porous glass bodies. The affinity of thermitase for the immobilized $-Ala_2-PheCH_3$ ligand linked to Divicell via an ε-aminocaproic acid (Aca) spacer is about two orders of magnitude lower than its affinity for the soluble inhibitor benzyloxycarbonyl–$Ala_2-PheCH_3$ and one order of magnitude lower than its affinity for Ac–Aca–$Ala_2-PheCH_3$. In comparison with affinity gels made with commercial CH-Sepharose 4B, the Divicell gels possess higher concentrations of ligands and have a higher stability.

INTRODUCTION

Peptidyl methyl ketones are potent reversible inhibitors of serine and cysteine proteases [1–5]. They are easy to synthesize, they have a higher chemical stability than other reversible peptide inhibitors (*e.g.* aldehydes) and their amino acid sequence may be adapted to the specificity of a defined enzyme. In an earlier paper [6] we introduced peptidyl methyl ketones as a new type of ligands in affinity chromatography of serine proteases. However, a number of questions remained unanswered (for instance the completeness of the enzyme elution, the long-term stability of the gels and the inhibition constant of the immobilized peptidyl methyl ketone are unknown).

In this work, the properties of the affinity gel Divicell–Aca–$Ala_2-PheCH_3$ (Aca = ε-aminocaproic acid) are discussed in comparison with

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the Sepharose 4B–Aca–Ala_2–$PheCH_3$ gel. Adsorption and desorption studies on both affinity gels were performed with the enzymes α-chymotrypsin, the subtilisins Carlsberg, BPN′ and DY and thermitase, as well as proteinase K.

Divicell, a macroporous bead cellulose with excellent flow parameters, was tested as support of affinity gels since it seems to possess some advantages in comparison with Sepharose 4B [7,8]. The inhibition constant (K_i) of the immobilized tripeptidyl methyl ketone –Ala_2–$PheCH_3$ in its reaction with thermitase was estimated and compared with the K_i values for the reaction of thermitase with the soluble inhibitors Z–Ala_2–$PheCH_3$ (Z = benzyloxycarbonyl) and Ac–Aca–Ala_2–$PheCH_3$. This last inhibitor should simulate the molecular structure of the spacer–tripeptidyl methyl ketone part of the affinity gel.

The completeness of the elution of the bound enzyme in the presence of isopropanol was examined by affinity chromatographic studies with ^{14}C-labelled acetyl-thermitase.

The separation of two related proteinases from a mixture by stepwise elution with increasing isopropanol concentration from the affinity gel Divicell–Aca–Ala_2–$PheCH_3$ was performed with a model mixture of pure subtilisin DY and pure thermitase.

Later, the paper describes the use of Divicell–Aca–Ala_2–$PheCH_3$ for the purification of thermitase from a crude extract. Considering the specificity of the cysteine proteinase papain, the affinity gels Divicell–Aca–Phe–$AlaCH_3$ and Divicell–β-Ala–Phe–$AlaCH_3$ were synthesized and adsorption and desorption studies on these gels were undertaken with papain.

EXPERIMENTAL

Inhibitors

Z–Ala_2–$PheCH_3$ was prepared according to Fittkau and Jahreis [1]. Ac–AcaOH was synthesized from ε-aminocaproic acid via H-AcaOMe × HCl and Ac–AcaOMe. Direct reaction of ε-aminocaproic acid with anhydrous acetic acid in pyridine as solvent produced by-products that could not be eliminated.

H–AcaOMe × HCl was obtained from the reaction of ε-aminocaproic acid with thionyl-chloride in water-free methanol [9] (yield, 97%; m.p. of the white needles 120–123°C; TLC, $CHCl_3$–CH_3OH, 9:1).

H–AcaOMe × HCl was acetylated in the presence of triethylamine with an excess of acetic anhydride. A light-brown oil was obtained which could not be induced to crystallize (yield, 40%; TLC, $CHCl_3$–CH_3OH, 9:1).

Ac–AcaOMe was saponified with 1 *M* NaOH within a few minutes to give white crystals of Ac–AcaOH (yield, 59%; m.p. 101–104°C; TLC, $CHCl_3$–CH_3OH, 9:1).

Ac–AcaOH was coupled with H–Ala_2–$PheCH_3$ × HCl using the mixed anhydride method to give a white powder of the tetrapeptidyl methyl ketone Ac–Aca–Ala_2–$PheCH_3$ [yield, 77%; m.p. 197–200°C; TLC, $CHCl_3$–CH_3OH, 5:1; CH_3OH–CH_3COOH–water, 2:1:2 (1% in ethyl acetate); $[\alpha]_D^{25}$ −23.6° (1% in ethyl acetate); HPLC RP-8 LiChrospher (Merck); elemental analysis for $C_{24}H_{36}N_4O_5$: C found 62.05 (calculated 62.59), H 7.88 (7.88), N 11.99 (12.16), O 18.08 (17.37)].

The purity of all intermediate compounds was controlled by TLC, by the melting point and by the optical activity; all final products were additionally characterized by elemental analysis, HPLC (RP-8 column, acetonitrile–water–0.1% trifluoroacetic acid system) and ^{1}H NMR spectroscopy. All compounds were purified to homogeneity.

In the case of Z–Ala_2–$PheCH_3$ and Ac–Aca–Ala_2–$PheCH_3$ two-dimensional ^{1}H COSY (correlated spectroscopy) spectra were also recorded using a Varian Unity 500 spectrometer (USA). The spectra were recorded in 256 experiments and a matrix of 256 × 2000 data points. As an example, the COSY spectrum of Ac–Aca–Ala_2–$PheCH_3$ is given in Fig. 1.

In order to distinguish between the ^{1}H signals of both alanine residues in the compounds Z–Ala_2–$PheCH_3$ and Ac–Aca–Ala_2–$PheCH_3$ NOE (nuclear Overhauser effect) difference spectra were recorded. The NOE experiments were performed with samples degassed repeatedly (five freeze–pump-through cycles). In all experiments the temperature was 25°C. The following proton chemical shifts with respect to internal tetramethylsilane were found for the three final products.

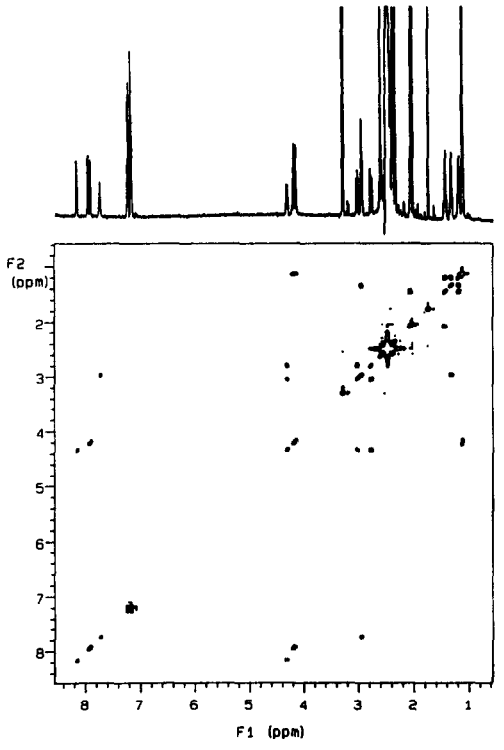

Fig. 1. Two-dimensional correlated 500-MHz proton NMR spectrum of Ac–Aca–Ala_2–$PheCH_3$ in [$^2H_6$]DMSO (15.8 mg/ml). A contour plot is shown, the one-dimensional spectrum on the diagonal, and the off-diagonal cross-peaks occur at the intersection of chemical shifts of two J-coupled spins. The one-dimensional ^1H NMR spectrum is shown at the top of the contour.

Z–Ala(2)–Ala(1)–PheCH₃. $PheCOCH_3$ (2.04 ppm); Phe (CαH 4.34; CβH 2.79 and 3.06; C_6H_5 7.16–7.25; NH 8.21); Ala(1) (CαH 4.22; CβH 1.16; NH 7.95); Ala(2) (CαH 4.03; CβH 1.16; NH 7.45); Z (CH_2 5.00; C_6H_5 7.33).

Ac–Aca–Ala(2)–Ala(1)–PheCH₃. $PheCOCH_3$ (2.04 ppm); Phe (CαH 4.33; CβH 2.79 and 3.04; C_6H_5 7.16–7.26; NH 8.16); Ala(1) (CαH 4.14–4.24; CβH 1.12–1.15; NH 7.97); Ala(2) (CαH 4.14–4.24; CβH 1.12–1.15; NH 7.93); Aca (CαH 2.07; CβH 1.42–1.48; CγH 1.31–1.37; CδH 1.18–1.23; CεH 2.94–2.99; NH 7.75); Ac (CH_3 1.76).

Z–Phe–AlaCH₃. $AlaCOCH_3$ (1.98 ppm); Ala (CαH 4.23; CβH 1.75; NH 8.42); Phe (CαH 4.26; CβH 2.74 and 2.99; C_6H_5 7.14–7.33; NH 7.54); Z (CH_2 4.93; C_6H_5 7.29).

Affinity gels

Divicell activated with 5-norbornene-2,3-dicarboximido carbonochloridate (30–34 μmol of activated OH groups per ml of gel) was a gift from Dr. H.-F. Boeden (Central Institute of Molecular Biology, Berlin, Germany) and Dr. R. Müller (Leipziger Arzneimittelwerke, Leipzig, Germany). It was suspended in 0.1 *M* sodium tetraborate buffer, pH 8.0, and coupled with ε-aminocaproic acid and β-alanine with stirring overnight. The amino acids were added in 30- or 25-fold molar excess, respectively, to the activated groups of the support [10]. Residual activated hydroxyl groups were bound with 1 *M* ethanolamine.

Titration of Divicell–AcaOH and Divicell–β-AlaOH was performed with 0.9 *M* NaOH in a TTT 2 pH-stat autotitrator (Radiometer, Copenhagen, Denmark). Concentrations between 17 and 21 μmol of carboxyl groups per ml of sedimented Divicell–AcaOH (depending on the charge) and 15 μmol of carboxyl groups per ml of Divicell–β-AlaOH were determined. In contrast, for commercial CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) 13 μmol of carboxyl groups per ml of gel were titrated.

The N-terminal free peptidyl methyl ketone hydrochlorides were coupled to the support–spacer conjugate with an excess of N-ethyl-N'-(3-dimethylamino-)propyl carbodiimide × HCl (Serva, Heidelberg, Germany) at pH 4.5–5.0 with stirring overnight at room temperature. The peptidyl component was added in a three- to six-fold molar excess compared with the spacer carbonyl groups. For instance, 11 ml of Divicell–AcaOH containing 231 μmol of carboxyl groups were coupled with 237 mg (= 693 μmol) of H–Ala_2–$PheCH_3$ × HCl freshly prepared from Z–Ala_2–$PheCH_3$ in 20 ml of water, pH 4.7, in the presence of 228 mg (= 1.19 mmol) of carbodiimide hydrochloride. Residual carboxyl groups were blocked with 1 *M* ethanolamine in the presence of carbodiimide.

The affinity gels were characterized by amino acid analysis with an Analysator 339 (Microtechno, Prague, Czech Republic) after total hydrolysis (boiling for 24 h with 6 *M* HCl under reflux). The amino acid compositions of the affinity gels are summarized in the following.

Sepharose 4B–Aca–Ala₂–PheCH₃. Aca 8.6 μmol per ml of gel (100%); Ala (2) 6.0 (70%); $PheCH_3$ 5.4 (63%); ethanolamine not determined.

Divicell–Aca–Ala₂–PheCH₃. Aca 14.1 μmol per ml of gel (100%); Ala (2) 8.4 (60%); $PheCH_3$ 8.6 (61%); ethanolamine 0.9 (6%).

Divicell–Aca–Phe–AlaCH₃. Aca 17.0 μmol per ml of gel

(100%); Phe 8.0 (47%); $AlaCH_3$ 7.4 (44%); ethanolamine 3.4 (20%).

Divicell-β-Ala-Phe-AlaCH$_3$. β-Ala 10.8 μmol per ml of gel (100%); Phe 4.0 (37%); $AlaCH_3$ 4.0 (37%); ethanolamine 2.3 (21%).

All gels were stored at 4°C in 0.1 *M* phosphate buffer, pH 6.0, containing 0.5 or 1.0 *M* NaCl and 0.02% NaN_3.

Substrates

Suc–Ala_2–PhepNA (Suc = succinyl; pNA = *para*-nitroanilide), a chromogenic substrate of serine proteinases with chymotryptic specificity, and Z–Phe–ArgNMec (NMec = N-methylcoumaryl amide), a cysteine proteinase substrate with a fluorescent end group after hydrolysis, were both synthesized in our laboratory according to the method of Brömme and co-workers [4,11].

Enzymes

Thermitase as a crude preparation and purified by adsorption on porous glass bodies was obtained from Dr. U. Kettmann (Institute of Physiological Chemistry, Martin Luther University Halle-Wittenberg, Halle, Germany). Thermitase purified by isoelectric focusing was a gift from Dr. W.E. Höhne (Institute of Biochemistry, Humboldt University Berlin, Germany). The subtilisins Carlsberg, Novo and DY were gifts from Professor N.C. Genov (Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria). α-Chymotrypsin and papain (crystal suspension) were purchased from Boehringer (Mannheim, Germany). Active site titrations were carried out with *trans*-cinnamoylimidazol according to Schonbaum *et al.* [12] on a Shimadzu UV 300 spectrophotometer (Japan).

The enzymatic activity of the serine proteinases against Suc–Ala_2–PhepNA in 0.1 *M* Tris–HCl buffer, pH 8.2, was recorded with an Eppendorf photometer (Germany). The activity of papain against Z–Phe–ArgNMec in 0.1 *M* Tris–HCl buffer, pH 7.5, was monitored on a Perkin-Elmer 1000 M fluorimeter (Germany).

The preparation of ^{14}C-labelled thermitase was performed as follows. A 2.5-mg aliquot of thermitase was dissolved in 500 μl of 0.1 *M* phosphate buffer (pH 7.5, 0.5 *M* NaCl) containing a 1.54-fold excess of Z–Ala_2–$PheCH_3$ and 1% (v/v) dimethyl sulphoxide (DMSO). At intervals of 10 min, three 1-μl aliquots (total: 32 μmol with a radioactivity of about 13.5 MBq) of ^{14}C-labelled acetic anhydride were added. The pH was adjusted to 7.5–7.6 with 0.92 *M* NaOH. After addition of the last aliquot of acetic anhydride the solution was allowed to react for 1 h at room temperature. In order to separate the acetylated enzyme from acetic acid, peptidyl methyl ketone and DMSO, gel filtration on Sephadex G-25 column (Pharmacia) was performed (column 43 × 1.2 cm; 0.1 *M* phosphate buffer, pH 7.5, 0.5 *M* NaCl; flow-rate 9 ml/h). A 100-μl volume of the fraction that contained the highest concentration of acetylated protein was directly applied to the affinity gel Divicell–Aca–Ala_2–$PheCH_3$ (column 3.5 × 0.7 cm) equilibrated with 0.1 *M* Tris–HCl buffer, pH 7.5, 0.5 m*M* calcium acetate.

Affinity chromatography

Simple binding and retention studies were carried out with pure enzymes and gel volumes between 1.1 and 1.4 ml (column length 4.0–16.0 cm, diameter 0.4–0.7 cm).

For the determination of the binding capacities and the K_i value of the immobilized tripeptidyl methyl ketone –Ala_2–$PheCH_3$, a small column containing 60 μl of affinity gel (1.30 × 0.25 cm) was used. The gels were equilibrated with 0.1 *M* Tris–HCl buffer, pH 7.8, 0.5 m*M* Ca^{2+}, before applying the enzymes. After loading 1.0–1.2 mg of enzyme dissolved in 200 μl of the buffer described above, the gels were washed with buffer containing 0.5 *M* then 1 *M* NaCl to break down and to determine non-specific adsorptions of the protein.

In order to elute the enzymes different amounts of isopropanol (10–50%, v/v) were added to the buffer. The flow-rate was adjusted to 2–3 ml/h.

The adsorption properties of the unmodified matrix as well as of the affinity gels against non-specific proteins were tested with haemoglobin.

The preparation of thermitase from prepurified crude material is described in the legend to Fig. 3. The fractions containing the purified

enzyme were combined and desalted by ultrafiltration in an Amicon cell (USA) with a YM 10 membrane. After this procedure thermitase was lyophilized.

All investigations using pure enzymes were performed at room temperature. The preparation of thermitase from crude material was performed at 4°C.

Kinetic parameters

The K_i values of the soluble inhibitors were determined as described by Fittkau *et al.* [2].

The K_i for the reaction of the tripeptidyl methyl ketone $-Ala_2-PheCH_3$ immobilized on Divicell via Aca with thermitase was estimated by modified frontal analysis [13]. A concentrated thermitase solution (3.0 mg/ml) with a defined activity was continuously applied to a column containing 60 μl of affinity gel until the enzymatic activity in the eluate was the same as the activity of the applied solution and remained constant.

RESULTS AND DISCUSSION

For the subtilisins Carlsberg and DY, thermitase and proteinase K a strong adsorption on both affinity gels, Sepharose 4B–Aca–Ala_2–$PheCH_3$ and Divicell–Aca–Ala_2–$PheCH_3$, was obtained. Subtilisin BPN′ and α-chymotrypsin were retained by the Divicell gel much more than by the Sepharose affinity gel, but in the case of α-chymotrypsin not enough to use Divicell–Ala_2–$PheCH_3$ for purification procedures.

Desorption of the bound enzymes could be achieved as described by Stepanov *et al.* [14] and Van den Burg *et al.* [15] by addition of isopropanol to the mobile phase. The amounts of isopropanol needed for a complete and sharp elution of the different proteinases correlate with the inhibition constants of the soluble inhibitor of these enzymes, Z–Ala_2–$PheCH_3$ [3]. The lower the K_i value, the more isopropanol is necessary. For instance, to elute subtilisin BPN′ ($K_i = 1.3 \cdot 10^{-4}$ *M*) from the gel Divicell–Aca–Ala_2–$PheCH_3$ 10% (v/v) isopropanol in the elution buffer is sufficient, but for the elution of thermitase ($K_i = 3.0 \cdot 10^{-7}$ *M*) at least 35% (v/v) isopropanol is needed. The other subtilisins as well as proteinase K, possessing inhibition constants between both these K_i values, are eluted sharply with isopropanol concentrations of 15–25% (v/v). Therefore, a 1:1 molar model mixture of pure subtilisin DY and pure thermitase could be separated by stepwise elution from the affinity gel Divicell–Aca–Ala_2–$PheCH_3$ using buffers containing 15% and 35% (v/v) isopropanol, respectively (see Fig. 2). However, since 15% isopropanol in the elution buffer causes a desorption of small amounts of thermitase, the eluted subtilisin DY is contaminated with it.

Using the Sepharose gel, in general lower isopropanol concentrations in the elution buffer are sufficient to elute the different enzymes, indicating weaker enzyme–ligand interactions [6]. The recovery of the enzymatic activity after affinity chromatography amounted in all cases to more than 85%.

The use of isopropanol as eluent has the advantage of enzyme stabilization. The enzymatic activity decreases rapidly with increasing isopropanol concentration. Autolysis is suppressed [15]. However, this inactivation is completely reversible as it could be shown after dilution of the alcohol to be less than 1% (v/v). Treating thermitase with a higher concentration of isopropanol even induces an increase in activi-

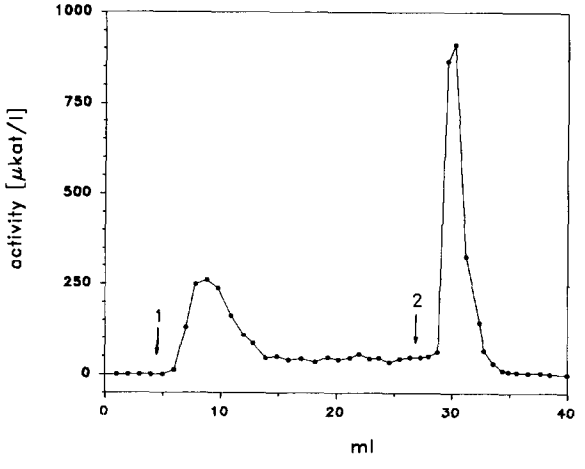

Fig. 2. Stepwise elution of subtilisin DY and thermitase from the affinity gel Divicell–Aca–Ala_2–$PheCH_3$. Conditions: 0.1 *M* Tris–HCl buffer, pH 7.8, 0.5 m*M* Ca^{2+}, 1 *M* NaCl, (1) 10% (v/v) and (2) 35% (v/v) isopropanol. Substrate: Suc–Ala_2–PhepNA. Column: 4.5 × 0.6 cm. Flow-rate: 3 ml/h.

ty after dilution [6]. In order to prove the completeness of elution, pure thermitase dissolved in buffer was acetylated with ^{14}C-labelled acetic anhydride in the presence of an excess of Z–Ala_2–$PheCH_3$. The peptidyl methyl ketone reversibly inhibits more than 99% of the enzyme activity and in this way prevents acetylation of active-site amino acid residues.

The inhibitor and the excess of acetylating reagent were eliminated by gel filtration on a Sephadex G-25 gel before applying 100 μl of the most radioactive protein fraction to an affinity column filled with Divicell–Aca–Ala_2–$PheCH_3$. Only 17% of the applied radioactivity appeared with the front; however, no enzymatic activity against Suc–Ala_2–PhepNA could be determined in these fractions. In the fractions containing 40% (v/v) isopropanol, 74% of the radioactivity and all the enzymatic activity appeared. Altogether, a recovery of 93% radioactivity and 87% enzymatic activity could be observed.

These investigations indicate that thermitase and —because of the recovery of enzymatic activity of between 85% and 95% in experiments with the other mentioned proteases— related enzymes could be completely eluted from affinity gels with peptidyl methyl ketone ligands by addition of isopropanol to the eluent.

In order to test the behaviour of an inert protein at the affinity gels Divicell- or Sepharose 4B–Aca–Ala_2–$PheCH_3$ haemoglobin was applied to the affinity columns. In neither case could any retention be observed. Therefore, haemoglobin could be used to determine the dead volume of a column system.

The pure support materials also retain neither haemoglobin nor the subtilisin proteinases.

The binding capacities of Sepharose 4B- and Divicell–Aca–Ala_2–$PheCH_3$ were estimated with thermitase. Whereas for the Sepharose gel a capacity of 1.7 μmol of enzyme per ml of sedimented gel was determined, for the Divicell gel a maximal adsorption of 1.4 μmol of enzyme per ml gel was found.

Considering the real concentrations of ligands, only about 28% and 17%, respectively, of the present ligands are able to bind active enzyme molecules. The difference in the binding capacities between the two gels can only be explained by steric effects.

When the Sepharose affinity gel was used more than 20–25 times (well below the capacity limit), changes in the adsorption and desorption properties were observed. The basic enzymatic activity measured in the eluate during the washing procedure —which is due to the equilibrium between bound and free enzyme and, in general, very low for tight-binding proteases— increases, and lower isopropanol concentrations in the elution buffer are sufficient for elution. The same effects could be observed if Sepharose 4B–Aca–Ala_2–$PheCH_3$ was stored at 4°C for 10 months. In this case, the capacity decreased by more than 40% [6].

In comparison, the gel Divicell–Aca–Ala_2–$PheCH_3$ seems to be much more stable than the Sepharose gel. Storage for more than 2 years and use far below the capacity limit does not cause changes in the gel properties.

One reason for the higher stability of the Divicell gel is the type of bonding between support and spacer. The Aca in the Divicell gel is bound to the support via urethane bonding, which is much more stable than the isourea bonding found in commercial CH-Sepharose [8]. Incubation of the affinity gel Sepharose 4B–Ala_2–$PheCH_3$ with a more concentrated enzyme solution for 2 days leads to the hydrolysis of the bonds between the two alanine residues and between spacer and ligand found with amino acid analysis [6].

The experiment that was performed to determine the binding capacity of the affinity gel Divicell–Aca–Ala_2–$PheCH_3$ was also used to estimate the inhibition constant, K_i, of the immobilized tripeptidyl methyl ketone according to the equation:

$$K_i = c_E c_I / c_{EI}$$

Under conditions of saturation c_E is equal to the enzyme concentration of the applied solution, c_{Eo}, c_{EI} is given by the binding capacity of the gel and c_I corresponds to the concentration of uncomplexed ligands involved in the equilibrium between free and bound enzyme.

The concentration of the free ligands could not be determined from the experiments but should be much lower than the difference between the total concentration of ligands and the concentration of the ligands complexed with enzyme,

because not all ligands are accessible to the enzyme molecules. Steric hindrance should be taken into account.

Therefore, K_i was calculated for a range of c_I from 1% to 13% of the total ligand concentration assuming that only 18–30% of immobilized ligands are able to interact with the enzyme. With a total ligand concentration of 8.5 μmol per ml of gel, an enzyme concentration $c_{Eo} = 7.8 \cdot 10^{-5}$ *M* in the interstitial volume of the gel and a maximal adsorption of 1.4 μmol of thermitase per ml of gel, a K_i value between 0.4 and $6 \cdot 10^{-5}$ *M* was found. In comparison, the K_i of the soluble thermitase inhibitor Z–Ala_2–$PheCH_3$ amounted to $3.0 \cdot 10^{-7}$ *M* [3]. Since this value takes into account the hydrophobic interactions of the benzyloxycarbonyl residue with the protein, the inhibitor Ac–Aca–Ala_2–$PheCH_3$ was synthesized to simulate the structure of the spacer-bound tripeptidyl methyl ketone better than the Z–Ala_2–$PheCH_3$ inhibitor.

In the case of thermitase, the K_i for Ac–Aca–Ala_2–$PheCH_3$ was determined to be $1.7 \cdot 10^{-6}$ *M*, which is about one order of magnitude greater than for the Z-protected inhibitor and about one order of magnitude smaller than for the immobilized peptidyl methyl ketone. It can be concluded that the kinetic constants of soluble peptidyl methyl ketones, and especially of inhibitors that contain the spacer at the N-terminus, are important in determining optimal ligands for affinity chromatography, although the conformational structure of the inhibitor may change during immobilization.

The affinity gel Divicell–Aca–Ala_2–$PheCH_3$ was used to purify thermitase in one step from a crude preparation. A concentrate of the culture medium of *Thermoactinomyces vulgaris* was prepurified by gel filtration on Sephadex G-75 and then directly applied to a column containing the affinity gel. After washing, the pure enzyme could be eluted with a sharp peak by adding 40% (v/v) isopropanol to the buffer. The elution diagram is shown in Fig. 3. After ultrafiltration and lyophilization the enzyme was characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), protein spectrum, specific activity against Suc–Ala_2–PhepNA and active site titration with *trans*-cinnamoylimidazol. Similar results can be obtained if thermitase is purified with Sepharose–Aca–Ala_2–$PheCH_3$ [6].

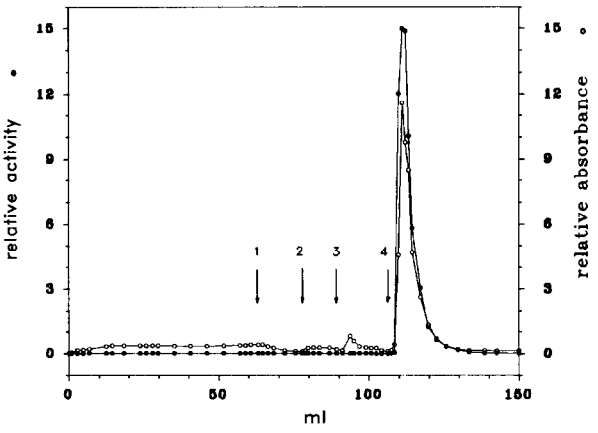

Fig. 3. Chromatography of thermitase on a column filled with the affinity gel Divicell–Aca–Ala_2–$PheCH_3$. Relative absorbance at 280 nm (○) = absorbance of the eluate/absorbance of the applied solution; relative activity (●) = activity of the eluate/activity of the applied solution. Conditions: Sephadex G-75 prepurified thermitase in 65 ml of 0.1 *M* ammonium acetate, pH 7.5, 0.5 m*M* Ca^{2+}. Arrows indicate the change of eluent: 1 = 0.1 *M* ammonium acetate, pH 7.5, 0.5 m*M* Ca^{2+}; 2 = 0.1 *M* Tris–HCl buffer, pH 7.8, 0.5 m*M* Ca^{2+}; 3 = 0.1 *M* Tris–HCl buffer, pH 7.8, 0.5 m*M* Ca^{2+}, 1 *M* NaCl; 4 = 0.1 *M* Tris–HCl buffer, pH 7.8, 0.5 m*M* Ca^{2+}, 1 *M* NaCl, 40% (v/v) isopropanol. Substrate: Suc–Ala_2–PhepNA. Column: 5.0 × 0.6 cm. Flow-rate: 12 ml/h (1 and 2), 5 ml/h (3).

Comparisons with thermitase fractions purified by isoelectric focusing [16] and adsorption on porous glass bodies [17] indicated that thermitase purified by affinity chromatography with the –Ala_2-$PheCH_3$ ligand as described has the same quality as the other preparations, for instance the electropherograms are almost the same for all enzyme charges (Fig. 4).

Specific activities between 1.40 and 1.98 μkat/mg and active amounts of total protein between 75% and 93% were determined for all thermitase fractions independently of their method of purification.

Peptidyl methyl ketones are effective inhibitors not only of serine proteinases but also of cysteine proteinases [4,5]. Inhibition constants in the micromolar range [4] lead to the assumption that peptidyl methyl ketones are also available as ligands in affinity chromatography of cysteine proteinases.

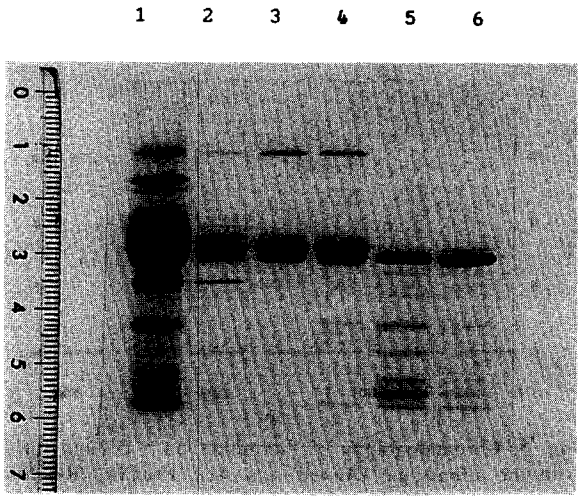

Fig. 4. SDS-polyacrylamide gel electrophoresis of thermitase. Collecting gel: 4.5% PAA, pH 6.8. Separation gel: 15.0% PAA, pH 8.8. Current: 40 mA, 2 h. In order to prevent autolysis during the boiling with SDS, thermitase was denatured with 20% trichloroacetic acid (1–4) and incubated at room temperature for 90 min with an eight-fold excess of the irreversible inhibitor Z–Ala_2–$PheCH_2Cl$ (5 and 6), respectively, before treatment with SDS. Lanes: 1 = Crude extract; 2 = after gel filtration on Sephadex G-75; 3 and 5 = after isoelectric focusing; 4 and 6 = after affinity chromatography with Divicell–Aca–Ala_2–$PheCH_3$.

Since papain, the best-known cysteine proteinase, possesses a specificity for substrates and inhibitors with aromatic amino residues in P_2 and relatively small amino acid residues in P_1 [18], the affinity gels Divicell–Aca–Phe–$AlaCH_3$ and Divicell–β-Ala-Phe–$AlaCH_3$ were synthesized and tested for papain binding.

Regardless of the spacer length, both gels adsorb papain strongly. In both cases the proteinase could only be eluted sharply with 50% (v/v) isopropanol in the elution buffer. However, papain also binds to Sepharose 4B- and Divicell–Aca–Ala_2–$PheCH_3$, but for elution an isopropanol concentration of only 30% and 40% (v/v), respectively, is sufficient. The lower isopropanol concentration in the latter experiments indicates weaker interactions between papain and the tripeptidyl methyl ketone ligand than between papain and the ligand –Phe–$AlaCH_3$. The recovery of enzymatic activity was 70–80% in all experiments.

Summarizing all investigations, it can be concluded that peptidyl methyl ketones are in general useful as ligands in affinity chromatography of serine and cysteine proteinases. The separation may be specified by adapting the amino acid sequence of the ligand to the specificity of the enzyme, which should be purified. Elution may be performed by use of an organic solvent that reduces autolysis of the proteases but influences the activity only reversibly. Because of its higher stability and higher ligand concentrations Divicell should be preferred over Sepharose as a support of the affinity gel.

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