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Purification of cathepsin B by a new form of affinity chromatography

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Human cathepsin B was purified by affinity chromatography on the semicarbazone of Gly-Phe-glycinal linked to Sepharose 4B, with elution by 2,2′-dipyridyl disulphide at pH 4.0. The product obtained in high yield by the single step from crude starting material was 80–100% active cathepsin B. The possibility that this new form of affinity chromatography may be of general usefulness in the purification of cysteine proteinases is discussed.

INTRODUCTION

Methods so far described for the affinity chromatography of cysteine proteinases all have disadvantages: some are applicable to only a few cysteine proteinases, others do not provide for separation of cysteine proteinases one from another, and most involve prolonged exposure of the enzymes to reducing conditions in which many are unstable. The new form of affinity chromatography described in the present paper for the purification of cathepsin B separates this enzyme from other cysteine proteinases, and keeps the active site protected most of the time. The ligand is a reversible inhibitor, Gly-Phe-GlySc, and elution is by specific blocking of free enzyme, in equilibrium with the gel, by 2,2′-dipyridyl disulphide.

EXPERIMENTAL

Z-Gly-Phe-GlySc and Gly-Phe-GlySc

Z-Gly-Phe-Gly-H was synthesized by a modification of the method of Mattis *et al.* (1977). Z-Phe-ONp (4.21 g, 10 mmol) in 10 ml of dimethylformamide was allowed to react overnight with an excess of aminoacetaldehyde dimethyl acetal (2.0 g). The solution was poured into a mixture of water and ethyl acetate, and the organic layer was washed with 1 M-NH_3, 1 M-HCl, then three times with 10% (w/v) $NaHCO_3$, and dried over $MgSO_4$. Evaporation *in vacuo* gave 3.38 g (88%) of Z-Phe-Gly-dimethyl acetal. T.l.c. on silica gel G (Merck) in methanol/chloroform (1:24, v/v) gave R_F 0.5. The benzyloxycarbonyl group was removed from Z-Phe-Gly-dimethyl acetal (3.7 g, 9.6 mmol) by catalytic hydrogenation (650 mg of Pd/charcoal) at atmospheric pressure, in 175 ml of methanol for 2.5 h. The catalyst was removed by filtration, and the solvent was evaporated *in vacuo* to give a quantitative yield of the free amine. The amine was treated immediately with Z-Gly-ONp (3.22 g, 9.6 mmol) in 10 ml of dimethylformamide at 0 °C for 3 h, and then overnight at 20 °C. Unsymmetrical *NN*-dimethyl ethylenediamine (1 ml) was added, and the solution was stirred for 30 min and then worked up as described for the dipeptidyl derivative to give 3.5 g (82%) of Z-Gly-Phe-Gly-dimethyl acetal after purification by flash chromatography (Still *et al.*, 1978) over silica gel, with elution with 5% (v/v) methanol in chloroform.

The acetal was hydrolysed to the aldehyde as described by Mattis *et al.* (1977), by heating with 10.85 g of Dowex 50 resin (H^+ form) in acetone (45 ml)/water (30 ml) at 65 °C for 2 h. T.l.c. [silica get G; methanol/chloroform (1:24, v/v)] gave R_F 0.5. The solvent was removed *in vacuo*, and the tripeptide aldehyde was purified by flash chromatography as above.

The aldehyde was converted into the semicarbazone as described by Patel *et al.* (1983). The crude aldehyde was dissolved in 95% (v/v) ethanol, and semicarbazide hydrochloride (0.86 g) and sodium acetate (0.66 g) were added in 5 ml of water. The solution was refluxed for 20 min, the solvents were removed by evaporation *in vacuo*, and the residue was crystallized from ethanol/ethyl acetate to give 2.7 g (45% yield) of the pure semicarbazone, Z-Gly-Phe-GlySc: m.p. 129–130 °C; t.l.c. on silica in 10% (v/v) methanol in chloroform gave R_F 0.3. The n.m.r. ([$^2H_6$]dimethyl sulphoxide) spectrum was consistent with the product. Analysis: calculated for $C_{22}H_{26}N_6O_5$ (454.49) C, 58.14; H, 5.77; N, 18.49 (Found C, 57.87; H, 5.97; N, 18.55%).

Treatment of the tripeptide semicarbazone at 20 °C with the buffers used in affinity chromatography for 12 h did not form tripeptide aldehyde (as tested by t.l.c.). The aldehyde was formed, however, on reaction with methanol/acetic acid/formaldehyde, as described by Patel *et al.* (1983).

The benzyloxycarbonyl group was removed from Z-Gly-Phe-GlySc (2.9 g) dissolved in methanol (200 ml) by catalytic hydrogenation (725 mg of Pd/charcoal) at atmospheric pressure for 2–4 h. Evaporation *in vacuo* gave 1.9 g (100%) of the free amine: t.l.c. [butan-1-ol/acetic acid/water (4:1:2, by vol.)] gave R_F 0.3. The free amine was used without further characterization to prepare the affinity resin.

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with IUB–IUPAC Recommendations [Biochem. J. (1984) **219**, 345–373]. Additional abbreviations are: Ahx, 6-aminohexanoyl; Arg-H, argininaldehyde; Gly-H, glycinaldehyde (aminoacetaldehyde); GlySc, glycinaldehyde semicarbazone (aminoacetaldehyde semicarbazone); -NHMec, 4-methyl-7-coumarylamide.

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Sepharose–Ahx-Gly-Phe-GlySc

Activated CH-Sepharose 4B (Pharmacia) (5 g dry wt) was soaked overnight in 1 mM-HCl at 4 °C, and washed with 0.1 M-$NaHCO_3$. Gly-Phe-GlySc (130 mg) was dissolved in 25 ml of methanol, and 15 ml of 0.1 M-$NaHCO_3$, pH 8.0, was added. The activated Sepharose was agitated in the solution in a roller rack overnight at 20 °C. The gel was collected on a sintered-glass filter, washed with 50% (v/v) methanol and then water, agitated in 6% (v/v) ethanolamine adjusted to pH 9.0 (with HCl) for 4 h at 20 °C, washed in water and stored at 4 °C in 0.1% NaN_3.

Crude cathepsin B

This was prepared as described by Barrett (1973) from 600 g of human liver, as far as acetone fractionation. The material precipitated by 47–64% (v/v) acetone was collected by centrifugation, redissolved in 50 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA, and dialysed against the same buffer overnight at 4 °C. The brown solution (230 ml) was applied to the affinity medium (10 g of the damp gel).

Other proteinases

These were prepared and assayed as described in the references cited: cathepsin H (Schwartz & Barrett, 1980), cathepsin L (Mason *et al.*, 1985), chymopapain and papain (Buttle & Barrett, 1984) and actinidin (Brocklehurst *et al.*, 1981).

Use of the affinity medium

The solution of crude cathepsin B (see above) was made 2 mM with respect to dithiothreitol and stirred with Sepharose–Ahx-Gly-Phe-GlySc for 2 h at 20 °C. The gel was then washed on a sintered-glass funnel with 50 mM-sodium phosphate buffer, pH 6.0, containing 0.5 M-NaCl, and with 50 mM-sodium format buffer, pH 4.0, before being packed into a column (12 cm × 1 cm diam.). 2,2′-Dipyridyl disulphide (1.5 mM) in 50 mM-sodium formate buffer, pH 4.0, was run into the gel, the flow was stopped, and the column was left overnight at 4 °C. Elution with the 2,2′-dipyridyl disulphide solution was then resumed, fractions being collected and assayed for cathepsin B and protein. Active fractions were combined, and the protein was concentrated and transferred into 50 mM-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA by ultrafiltration on an Amicon YM-5 membrane.

The gel medium was stored in 0.10 M-Tris/HCl buffer, pH 8.0, containing 0.1% NaN_3 until re-used; the high pH value would be expected to inactivate and elute any traces of firmly bound cathepsin L.

Analytical methods

Enzyme assays and active-site titration were as described by Barrett & Kirschke (1981). Z-Arg-Arg-NHMec was used as substrate in assays for cathepsin B, 1 unit of activity corresponding to the formation of 1 μM of product/min under the standard conditions. The less-specific substrate Z-Phe-Arg-NHMec was used in active-site titrations of the pure enzyme. K_i values were determined as described by Mason et al. (1985) for leupeptin with cathepsin L, but in sodium phosphate buffer at pH 6.0 and 30 °C. SDS/polyacrylamide-gel electrophoresis was in 15% (w/v) gels with reduction (Bury, 1981).

Protein was determined by the Bio-Rad dye-binding assay (Bio-Rad Laboratories, Watford, Herts., U.K.). With pure cathepsin B, this assay gave results in close agreement with the Lowry method and direct A_{280} measurement used previously (Barrett, 1973), but the dye-binding assay was better suited to samples containing coloured material, dithiothreitol and 2,2′-dipyridyl disulphide. When samples contained 2,2′-dipyridyl disulphide, this was also included in the blanks, since it gave slight positive interference. For the purpose of calculating the molar concentration of solutions of cathepsin B, and the percentage purity, the M_r was taken as 27625 (Ritonja *et al.*, 1985).

Immuno-blots from electrophoresis gels were prepared on nitrocellulose paper as described by Davies & Barrett (1984). Antisera were raised by injection of rabbits with cathepsin B (Schwartz & Barrett, 1980) or cathepsin L (Mason *et al.*, 1985).

Samples for amino acid analysis were hydrolyzed in 6 M-HCl containing 1% phenol *in vacuo* for 24 h at 105 °C. The hydrolysates (freed of HCl) were run on the Micro Pak Hydrolysate column of a Varian LC 5000/Vista 402 h.p.l.c. system.

RESULTS

Values of K_i for cathepsin B were 3.4 μM and 63 μM for Z-Gly-Phe-GlySc and Gly-Phe-GlySc respectively.

The Sepharose–Ahx-Gly-Phe-GlySc medium was found by amino acid analysis to contain 3.8 μmol of phenylalanine and 3.4 μmol of glycine per g wet wt., showing the presence of about 3.6 μmol of the peptide ligand/g of gel (i.e. per ml bed volume). GlySc does not yield glycine in acid hydrolysis. The saturation capacity of the medium for cathepsin B was 1.8 mg, or 65 nmol, per ml of gel, so that it can be calculated that only about 2% of the bound ligand groups were interacting with enzyme molecules at saturation. This may have been due to steric hindrance by the gel matrix or by enzyme molecules already bound.

The results of use of the affinity medium are summarized in Table 1. There was a large increase in total activity during the acetone fractionation step, presumably due to removal of inhibitors. This prevents direct comparison of the overall yield with those in earlier work with different assay methods, but the recoveries of 60–80% from the affinity column compare very favourably with, for example, 7% recovery during the post-acetone stages in our original method (Barrett, 1973). Active-site titration of cathepsin B eluted from the affinity medium (as described in the Experimental section) showed it to be 80–100% active (three experiments). This also is much better than the older method, which gave 40–50% active material in a number of preparations in this Laboratory (D. J. Buttle, personal communication).

The eluted enzyme was analysed by SDS/polyacrylamide-gel electrophoresis with reduction. The result (Fig. 1) shows a major band of M_r 24000 and a diffuse minor band of M_r 5000, consistent with the heavy and light chains respectively of cathepsin B (Kirschke & Barrett, 1985). A further minor component of M_r 30000 could well correspond to a single-chain form of cathepsin B, and there were traces of higher-M_r bands, presumed to represent contaminants. The major band gave a

Table 1. Summary of results of purification of human cathepsin B

The procedure was as described fully in the text, the starting sample being an autolysate corresponding to 600 g of human liver.

Stage	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Purification factor	Yield (%)
Autolysate	2200	32.3	0.0147	(1)	(100)
Acetone fraction	202	69.0	0.341	23.2	213
Product from affinity medium	10.4	55.2	5.31	361	171

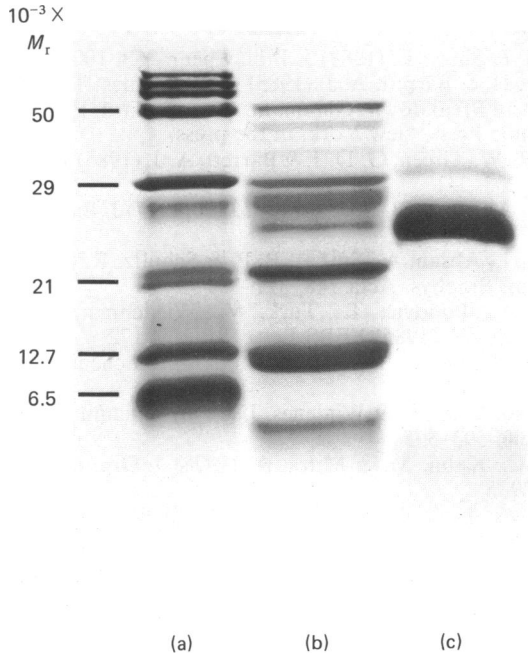

Fig. 1. SDS/polyacrylamide-gel electrophoresis of cathepsin B

The samples were: lane (a), M_r markers including IgG light chain, carbonic anhydrase, soya-bean trypsin inhibitor, bovine cytochrome *c* and aprotinin, for which M_r values are marked; lane (b), crude cathepsin B as applied to the affinity medium; lane (c), cathepsin B eluted from the gel with 2,2′-dipyridyl disulphide (very heavily loaded).

positive reaction with anti-(human cathepsin B) serum after blotting on to nitrocellulose.

The crude cathepsin B sample would have contained cathepsin H and cathepsin L. Cathepsin H was detectable in the material that passed through the column, and purified cathepsin H run separately was not significantly bound to the affinity medium. Cathepsin L was not detectable immunologically in the eluted fractions of cathepsin B, and a separate experiment with cathepsin L showed that the enzyme bound to the gel, but was not eluted during overnight treatment with 2,2′-dipyridyl disulphide. We therefore conclude that cathepsin B is efficiently separated from cathepsins H and L by the affinity medium, because cathepsin H is not adsorbed, and cathepsin L is not eluted during the period used for cathepsin B.

Three plant cysteine proteinases tested behaved like cathepsins H or L; thus chymopapain was not bound, whereas papain and actinidin were not eluted.

The affinity medium seemed to be re-usable indefinitely; one batch of gel used eight times showed no deterioration in performance.

In further experiments, not reported here in detail, attempts were made to use the affinity medium in conjunction with preliminary fractionation by $(NH_4)_2SO_4$ (Mason *et al.*, 1985), rather than acetone. Again, a high degree of purification was achieved, but an additional component of apparent M_r 40000, with no detected proteolytic activity, contaminated the final preparation of cathepsin B.

DISCUSSION

There is a need for new methods of affinity chromatography of cysteine proteinases. Of the methods so far described, the -Gly-Gly-(OBzl)Tyr-Arg ligand introduced by Blumberg *et al.* (1970) for papain has not proved to be applicable to other cysteine proteinases (Barrett, 1973). The aminophenylmercuric ligand introduced for papain by Sluyterman & Wijdenes (1970) is applicable to cathepsin B (Barrett, 1973), but does not bind cathepsin H efficiently, and has the disadvantage that elution with a thiol reagent leaves the enzyme in the activated state in which most cysteine proteinases are unstable (Barrett, 1973). The principle of covalent chromatography on 2,2′-dipyridyl disulphide-activated thiol–Sepharose described by Brocklehurst & Little (1973), and used for actinidin and cathepsin B (Brocklehurst *et al.*, 1981; Willenbrock & Brocklehurst, 1985), has been further developed by Evans & Shaw (1983) with a view to making it more specific for cysteine proteinases, but the selectivity of the method among cysteine proteinases is not established, and again the enzyme is eluted in its activated state.

The peptide aldehyde inhibitors resembling leupeptin (Ac-Leu-Leu-Arg-H) are attractive candidates for use as ligands in the purification of proteinases because of their high affinities, but aldehydes have the general disadvantage of being chemically highly reactive, and analogues of leupeptin have the particular disadvantage of carrying a positive charge that introduces unselective binding by ion exchange. The aldehydes bind both serine proteinases and cysteine proteinases, and no satisfactory elution scheme has been described for the cysteine proteinases.

Because of the limitations in the usefulness of available forms of affinity chromatography for cysteine proteinases, it will be of interest to discover whether the method that we have developed for cathepsin B can be adapted to other enzymes of this class. This form of affinity chromatography can be expected to be specific for

cysteine proteinases. Serine proteinases have some affinity for peptidyl semicarbazones (R. Beynon & A. J. Barrett, unpublished work), but the present method is made specific by use of 2,2′-dipyridyl disulphide to block free cysteine proteinase molecules in equilibrium with the immobilized complex, and thus displace them. Dipyridyl disulphide reacts very selectively with the active-site cysteine residues of cysteine proteinases at pH 4 (Brocklehurst & Little, 1973). The 2,2′-dipyridyl disulphide-blocked enzyme eluted from the column is in an inactive protected state, but is rapidly activated by dithiothreitol under the conditions of assay or active-site titration.

Chromatography on Sepharose–Gly-Phe-GlySc discriminates among cysteine proteinases. Cathepsin H and chymopapain were not efficiently bound, whereas cathepsin L, papain and actinidin could not be eluted within a reasonable time. The rate of displacement of the enzyme by 2,2′-dipyridyl disulphide should relate inversely to the K_i of the enzyme for the immobilized ligand, and other peptidyl semicarbazones of appropriate K_i may well be found for the purification of other cysteine proteinases.

In conclusion, the method described here represents an excellent single-column purification procedure for human cathepsin B, and the principle may be capable of extension to other cysteine proteinases.

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