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PHENYLBORONIC ACID AS A LIGAND FOR BIOSPECIFIC CHROMATOGRAPHY OF SERINE PROTEINASES

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SUMMARY

Via attachment of *p*-(ω-aminoethyl)phenylboronic acid to CH-Sepharose in the presence of water-soluble carbodiimide, a new sorbent for the biospecific chromatography of serine proteinases was obtained. The sorbent was shown to be suitable for the purification of subtilisin, α-chymotrypsin and trypsin. It is assumed that the serine hydroxyl group at the active site of the enzyme forms, with the boronic acid moiety of the ligand, a structure that imitates transition enzyme–substrate complex. The presence of glycerol selectively improves the binding of serine proteinases, presumably because of stabilization of the tetrahedral state of the boron atom. Direct isolation of subtilisin from a *Bacillus subtilis* cultural filtrate on phenylboronic acid-containing sorbent gives a virtually homogeneous enzyme (42-fold purification) in a nearly-quantitative yield.

INTRODUCTION

Boronic acid derivatives as inhibitors of serine proteinases were described for the first time by Antonov *et al.*[1], who postulated that the boronic acid residue formed a labile bond at the active site whereas the hydrocarbon moiety interacted with the hydrophobic binding site of the enzyme. Later, Koehler and Lienhard[2] suggested that the complexes with substituted boronic acids reproduced the structure of the transition state at the active site of serine proteinases. This assumption was confirmed by X-ray analysis[3]. It was found that the boronic acid residue formed a covalent bond with the serine residue at the active site. The high specificity of this interaction implied the possibility of its use for the biospecific chromatography of serine enzymes.

MATERIALS AND METHODS

CH-Sepharose was purchased from Pharmacia (Uppsala, Sweden) and α-chymotrypsin, bovine trypsin and subtilopeptidase A (subtilisin BPN') from Serva (Heidelberg, G.F.R.). Subtilisin inhibition with phenylmethylsulphonyl fluoride

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(PMSF) was performed as described earlier[4]. The following substrates were used for proteinase assay: N-benzoyl-D,L-arginine *p*-nitroanilide for trypsin (method 2 of Haverback *et al.*[5]), N-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide for α-chymotrypsin[6] and benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide for subtilisin[7]. The unit of activity was taken as the amount of the enzyme that splits, under specified conditions, 1 μmole of the substrate per minute.

Thin-layer chromatography (TLC)

TLC was performed on silica gel-coated plates in the following solvent mixtures: A = *n*-butanol–water (86:14); B = toluene–acetone (1:1); C = ethyl acetate; D = propanol-2–ammonia solution–water (7:1:2); and E = propanol-2–hydrochloric acid–water (17:4:5). The spots were rendered visible with iodine vapour[8] or by spraying with a 0.2% solution of diphenylcarbazone in methanol[9]. After spraying with the latter reagent, arylboronic acids appeared as red or violet spots on a pink background.

CHPB-Sepharose synthesis

p-Tolueneboronic acid (I) was synthesized by the reaction of *p*-tolylmagnesium bromide with trimethyl borate[10]. After recrystallization from water the yield was 41% (m.p. 243–245°; literature[11] m.p. 245°). The substance gave one spot on TLC with R_F = 0.89 in solvent A, 0.57 in solvent B and 0.31 in solvent C.

p-(ω-Bromomethyl)phenylboronic acid (II) was obtained by N-bromosuccinimide bromination of the side-chain in I[12]. The yield was 80% (m.p. 146°; literature[13] m.p. 165–168°) TLC gave R_F = 0.91 in solvent A, 0.57 in solvent B and 0.31 in solvent C.

The urotropin complex of II (III) was obtained in quantitative yield from freshly prepared II as described by Pichuzhkina *et al.*[14] and could be used for the following stage without purification. After treatment with hot water-saturated *n*-butanol, on TLC the substance gave one spot with R_F = 0.0 in solvent A, 0.10 in solvent B and 0.34 in solvent D.

p-(ω-Aminomethyl)phenylboronic acid (IV) was obtained by decomposition of III with hydrochloric acid. A 1.7-g amount of III was refluxed with 54 ml of ethanol and 20 ml of concentrated hydrochloric acid for 1 h, then the mixture was repeatedly evaporated to dryness *in vacuo* from ethanol. The residue was treated with hot, dry ethanol, ammonium chloride filtered off and the filtrate evaporated to dryness, giving 0.75 g of IV (yield 90%). The substance could be coupled with CH-Sepharose without further purification. For purification, 100 mg of IV were dissolved in sodium hydroxide solution at pH 11 and the solution was poured into a Dowex 1-X1 column (10 × 1 cm) that had been previously washed with 1 m*M* sodium hydroxide solution. The column was washed with 1 m*M* sodium hydroxide solution and water, then IV was eluted with 0.1 *M* hydrochloric acid. Pure IV showed one spot on TLC with R_F = 0.87 in solvent A, 0.17 in solvent D and 0.56 in solvent E.

CHPB-Sepharose, the product of the attachment of *p*-(ω-aminomethyl)phenylboronic acid to CH-Sepharose, was obtained as follows. To 2.5 ml of CH-Sepharose (containing about 35 mmole of COOH groups, as certified by the manufacturer) were added 25 mg of IV followed by 41.2 mg of N-cyclohexyl-N'-[2-(4-morpholinyl)ethyl]-carbodiimide *p*-toluenesulphonate. After dissolution of the reagents, the mixture

(pH 4.8) was kept overnight at 20°, then CHPB-Sepharose was collected on a glass filter and washed thoroughly with water. To determine the content of IV in CHPB-Sepharose, 0.1 ml of settled sorbent was treated with 5 ml of 6 *M* hydrochloric acid at 20° until the gel had dissolved, then the concentration of the ligand was calculated from the UV spectrum of the solution using the molar extinction coefficient ε_{225}^{M} = 8390 for IV in 6 *M* hydrochloric acid.

Chromatography on CHPB-Sepharose

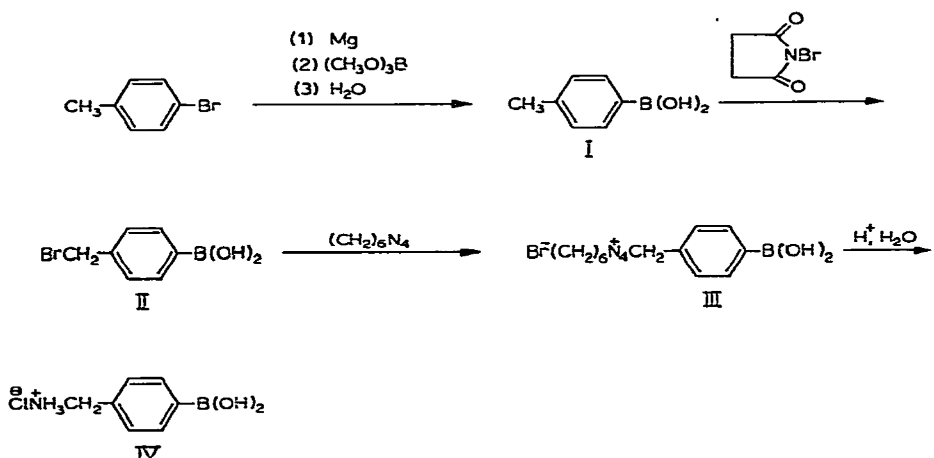
A CHPB-Sepharose column (25 × 1 cm) was equilibrated with 0.05 *M* phosphate buffer of appropriate pH or with 0.5 *M* glycerol solution in the same buffer. Sample volumes did not exceed 2 ml. The column was eluted with equilibrating buffer at 36 ml/h. The protein content was determined on the basis of the absorbance at 280 nm.

Isolation of subtilisin from Bacillus subtilis cultural filtrate

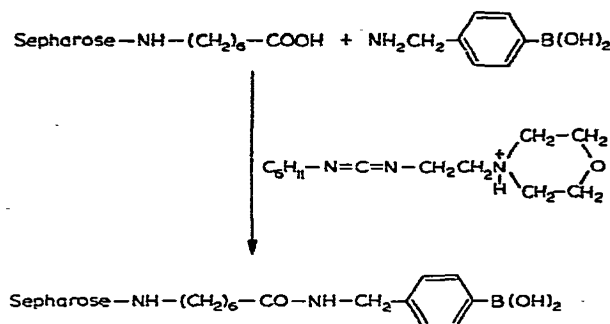
The cultural filtrate of *Bacillus subtilis* strain A-50 was concentrated to 5% of its volume by dialysis against Carbowax 6000, then dialysed against 0.5 *M* glycerol in 0.05 *M* phosphate buffer (pH 7.5). A 2-ml volume of this solution was applied on a 25-ml CHPB-Sepharose column equilibrated with the same buffer. Further operations were carried out as described above.

RESULTS AND DISCUSSION

p-(ω-Aminomethyl)phenylboronic acid was synthesized by the following reaction sequence:



p-(ω-Aminomethyl)phenylboronic acid was attached to CH-Sepharose (Sepharose derivative, containing ε-aminocaproic acid residues with free carboxyl groups) activated by water-soluble carbodiimide:



The sorbent obtained contained 5–10 μmole of the ligand per millilitre of swollen Sepharose, whereas 10–14 μmole of free carboxyl groups per millilitre were claimed by the manufacturer for CH-Sepharose. CHPB-Sepharose was tested as a sorbent for the covalent chromatography of serine proteinases.

Subtilisin BPN′, an extracellular serine proteinase of *Bacillus subtilis*, was not firmly bound but only retarded by the sorbent (Fig. 1a). The maximal extent of the retardation was attained at pH 7.5 (Fig. 2), which agreed fairly well with the data on the pH-dependence of the inhibition of serine proteinases by boronic acids[15,16]. Chromatography on CHPB-Sepharose at this pH permits the separation of the active subtilisin from inert proteins and also from PMSF-inhibited subtilisin not retarded by the column (Fig. 1c and e). This clearly shows the importance of the interaction of serine hydroxyl groups with the boronic acid moiety of the ligand for the sorption of subtilisin. The chromatography of a commercial subtilisin preparation on CHPB-Sepharose gave a 1.9-fold purification of the enzyme (Table I). Its specific activity was 1.82 units/mg when tested against the chromogenic substrate, benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide, which corresponds to essentially pure enzyme. A high recovery of the enzyme (81 %) was achieved.

TABLE I

CHROMATOGRAPHY OF PROTEINASES ON CHPB-SEPHAROSE

The sorbent was equilibrated and washed with 0.05 *M* phosphate buffer, pH 7.5.

Enzyme preparation	*Glycerol added (M)*	*Column volume (ml)*	*Protein applied (mg)*	*Specific activity (units/mg × 10^2)*		*Purification factor*	*Yield (%)*
				Initial	*Final*		
Subtilisin BPN′	0	25	4.25	94	182	1.9	81
Subtilisin BPN′	0.5	25	4.1	94	204	2.2	82
Subtilisin A-50 (cultural filtrate)	0.5	25	50	4.3	180	42	100
Trypsin	0	4	1	22	33	1.5	100
Trypsin	0.5	4	1	22	30	1.4	100
α-Chymotrypsin	0	4	1	2.5	4	1.6	80
α-Chymotrypsin	0.5	4	1	2.5	4	1.6	91

Pentaerythritol, which forms with phenylboronic acid at pH 7.5 a bicyclic complex[17] in which all three hydroxyl groups of the tetradentate boron atom are in covalent bonds with the alcohol, completely suppressed the interaction of subtilisin

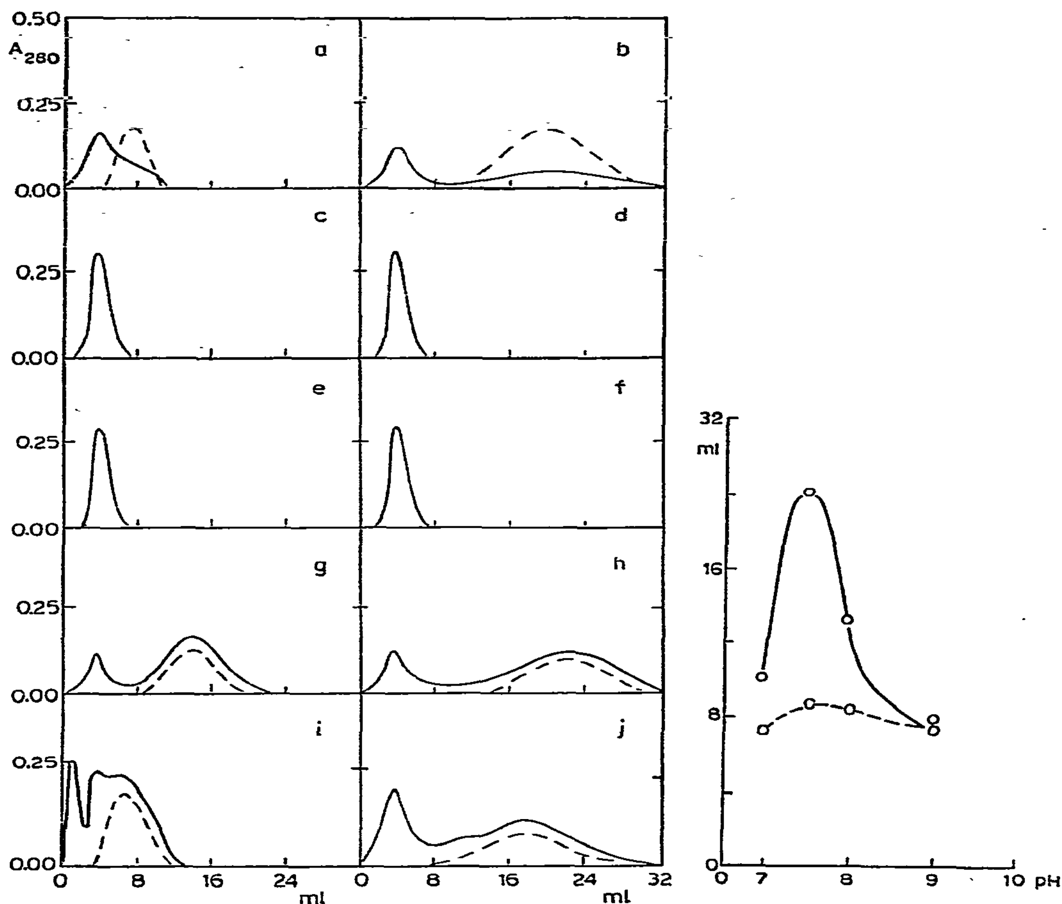


Fig. 1. Chromatography of proteins on CHPB-Sepharose. To a column containing 4 ml of CHPB-Sepharose, 0.1 ml of 1% protein solution was applied. The column was equilibrated and eluted with 0.05 *M* phosphate buffer (pH 7.5) or with 0.5 *M* glycerol in the same buffer. Solid line, absorbance at 280 nm (protein content); broken line, proteolytic activity in arbitrary units. (a) Subtilisin BPN'; (b) subtilisin BPN' in 0.5 *M* glycerol; (c) bovine serum albumin; (d) bovine serum albumin in 0.5 *M* glycerol; (e) PMSF-inhibited subtilisin BPN'; (f) PMSF-inhibited subtilisin in 0.5 *M* glycerol; (g) α-chymotrypsin; (h) α-chymotrypsin in 0.5 *M* glycerol; (i) trypsin; (j) trypsin in 0.5 *M* glycerol.

Fig. 2. pH dependence of subtilisin BPN' retention volume on CHPB-Sepharose. To a column containing 4 ml of CHPB-Sepharose, 0.05 ml of 1% subtilisin BPN' solution was applied. The points correspond to the maxima on the elution curves of the active enzyme. Broken line, column equilibrated and washed with 0.05 *M* phosphate buffer (pH 7.5); solid line, column equilibrated and washed with 0.5 *M* glycerol in the same buffer.

with CHPB-Sepharose. This result confirms that it is the hydroxyl groups of substituted phenylboronic acid that take part in the binding of the enzyme.

The chromatography of other serine proteinases (α-chymotrypsin and trypsin on CHPB-Sepharose gave similar results (Fig. 1g and i; Table I).

Whereas pentaerythritol blocked the binding of serine proteinases by CHPB-Sepharose, glycerol, on the other hand, enhanced the interaction of these enzymes

with the sorbent. Glycerol is known to form at pH 7.5 a complex with phenylboronic acid in which only two hydroxyl groups of the tetradentate boron atom participate in covalent bonds with the alcohol[17], leaving the third hydroxyl group free to interact with the hydroxyl group at the active site of serine proteinases. The complexes of these enzymes with CHPB-Sepharose are even more stable in 0.5 *M* glycerol, which results in increased retardation by the sorbent (Fig. 1b, h and j). Thus, in the presence of glycerol the retention volume of subtilisin BPN' is 2.5 times, of trypsin 2.2 times and of α-chymotrypsin 1.5 times as large as in its absence.

It has been shown that substituted boronic acid derivatives, when bound to serine proteinase, imitate the tetrahedral transition state of an enzyme–substrate complex[2,3]. One of its hydroxyl groups establishes a covalent bond with the serine residue of the active site, whereas the other two serve as acceptors of the hydrogen bonds with the donor groups belonging to so-called "oxyanion hole" of the enzyme (Fig. 3).

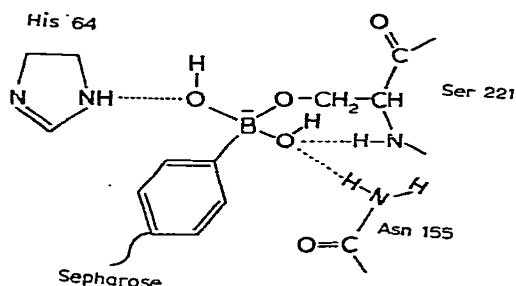


Fig. 3. Hypothetical structure of CHPB-Sepharose complex with the active site of a serine proteinase (amino acid residue numbering is given for subtilisin)[3]. Glycerol presumably binds with both free hydroxyl groups.

It appears that the formation of a phenylboronic acid–glycerol complex does not prevent the building of these hydrogen bonds. On the other hand, the stabilization of the tetrahedral state of the boron atom due to the interaction with glycerol even improves the stability of the enzyme–ligand complex. This hypothesis might explain the positive effect of glycerol on the binding of serine proteinases by CHPB-Sepharose (Fig. 1b, h and j). The presence of glycerol does not lower the selectivity of serine proteinase chromatography. Thus, PMSF-inhibited subtilisin BPN' was eluted in 0.5 *M* glycerol with the free volume of the column, together with the inert proteins (Fig. 1d and f). No shift in the pH optimum for retardation of serine proteinases was observed in glycerol.

The enhancement of the binding properties of CHPB-Sepharose in glycerol-containing solutions has been used for the direct isolation of subtilisin from *Bacillus subtilis* A-50 cultural filtrate. In a single step by filtration through a CHPB-Sepharose column, equilibrated with 0.5 *M* glycerol in 0.05 *M* phosphate buffer of pH 7.5 (Fig. 4), a subtilisin preparation was obtained with a specific activity of 1.8 units/mg, which can be compared with 0.94 units/mg for a commercial preparation of subtilisin BPN' and 1.82–2.04 units/mg for subtilisin BPN' purified on CHPB-Sepharose as described above (Table I). No interference from the components of the cultural filtrate was observed although the interaction of the boronic acid moiety with sugars present in the mixture could adversely affect the procedure. It appears that the content

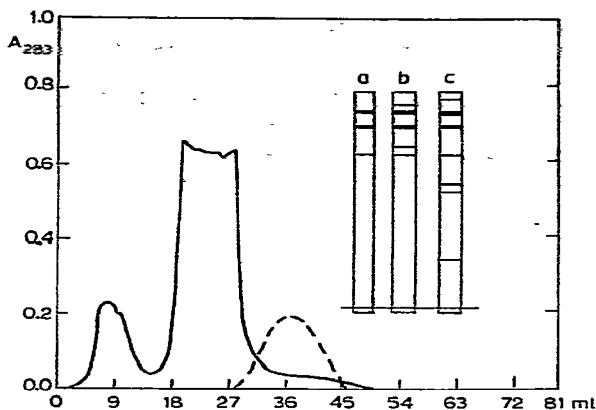


Fig. 4. Isolation of subtilisin from the cultural filtrate of *Bacillus subtilis* A-50. To a CHPB-Sepharose column (volume 25 ml), 2 ml of cultural filtrate were applied. The column was equilibrated and washed with 0.5 *M* glycerol in 0.05 *M* phosphate buffer (pH 7.5). Solid line, absorbance at 280 nm; broken line, proteolytic activity in arbitrary units. The inset shows the disc-electrophoresis pattern in 7.5% polyacrylamide gels (pH 8.9)[18]: (a) subtilisin BPN' purified on CHPB-Sepharose (see Fig. 1b); (b) subtilisin isolated from *Bacillus subtilis* A-50 cultural filtrate; (c) commercial subtilisin BPN'. Direction of movement, from the top to the bottom; the gels were stained with Coomassie Brilliant Blue R 250. The three most intense bands (R_F = 0.08, 0.16 and 0.30) correspond to the multiple forms of subtilisin BPN'[4].

of sugars, which is usually low at the end of logarithmic growth, was drastically reduced in the course of liquid culture analysis.

Hence, CHPB-Sepharose might be used as a specific sorbent for the chromatography of serine proteinases. It should be mentioned that for some enzymes of this type no satisfactory conditions were found that would permit their purification on CHPB-Sepharose (serine carboxypeptidase from *Aspergillus oryzae* and intracellular serine proteinase from *Bacillus subtilis*). Both of these enzymes possess isoelectric points near pH 4 and it would therefore be reasonable to assume that electrostatic interactions might be operative in the course of the chromatography of enzymes on CHPB-Sepharose, in addition to the specific binding discussed above. Such non-specific factors have to be taken into account and controlled. It appears that CHPB-Sepharose might be of value as a specific sorbent for various serine hydrolases.

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