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CARBOXYPEPTIDASE S-1 FROM PENICILLIUM JANTHINELLUM: ENZYMATIC PROPERTIES IN HYDROLYSIS AND AMINOLYSIS REACTIONS

by

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Carboxypeptidase S-1 from Pencillium janthinellum has been isolated by affinity chromatography and characterized. The enzyme activity is unusually stable in organic solvents, e.g. 80% methanol. The hydrolysis of peptide substrates is apparently dependent on three ionizable groups. One group, with pKa of 4.0-4.5, is a catalytically essential residue in its deprotonated form, and another group with a pKa of 6.5-7.0 functions in its protonated form, apparently as the binding site for the C-terminal carboxylate group of peptide substrates. The third group, with a pKa of 5.0-5.5, appears to be a carboxylic acid group located at the S_1 binding site. Deprotonation of this group to form a negatively charged carboxylate group has an adverse effect on the hydrolysis of substrates with hydrophobic amino acid residues at the P_1 position and a beneficial effect on the hydrolysis of substrates with the positively charged arginyl or lysyl residues at this position. The substrate preference of the enzyme is consequently pH dependent. At pH 6.0 (the optimum for ester hydrolysis) the enzyme is essentially specific for Bz-X-OMe substrates where X = Arg and Lys.

Using amino acids and amino acid amides as nucleophiles carboxypeptidase S-1 efficiently catalyses the formation of peptide bonds by aminolysis of peptides (transpeptidation reactions) and peptide esters provided that the substrate contains a basic amino acid residue at the P_1 position, e.g. Bz-Arg-OBu and Bz-Arg-Leu-OH. With several nucleophiles the fractions of aminolysis exceed those previously reported in similar studies with carboxypeptidase Y and malt carboxypeptidase II.

Abbreviations: Bicine = N,N-bis(hydroxyethyl)glycine; Bu = butyl; Bz = N-benzoyl; CPD-M_I, CPD-M_{II} and CPD-M_{III} = malt carboxypeptidases I, II and III, respectively; CPD-Y = carboxypeptidase Y; CPD-S-1 = penicillocarboxypeptidase S-1; EDTA = ethylene diamine tetraacetic acid; FA = furylacryloyl; Hepes = N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; HPLC = high performance liquid chromatography; Mes = 2-(N-morpholino)ethane sulfonic acid; Z = N-carbobenoxy. Other abbreviations of amino acids, amino acid derivatives and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature. The binding site notations for the enzymes is that of SCHECHTER and BERGER (20). Accordingly, the binding site for the C-terminal amino acids residue of the substrate is denoted S_1' and those for the amino acid residues in the amino-terminal direction away form the scissile bond are denoted $S_1, S_2 ..., S_n$. The substrate positions are all denoted $P_1', P_1, P_2 ... P_n$ in correspondence with the binding sites.

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1. INTRODUCTION

The ability of serine carboxypeptidases to catalyse elongation of peptides, exchange of C-terminal amino acid residues and incorporation of C-terminal amide groups into peptides and proteins by aminolysis reactions has been described in several reports. The fractions of aminolysis and the amounts of side-products produced is crucially dependent on how the substrate preference of the enzyme utilised corresponds to the sequence of the peptide synthesised. At present, such reactions have been described with carboxypeptidase Y (2, 3, 4, 6, 8, 9, 23, 24, 25), carboxypeptidases I and II from malt (10, 13) and carboxypeptidase II from wheat (21). Although the substrate preferences of these enzymes to some extent supplement each other the isolation of alternative serine carboxypeptidases with different specificities is desirable. It is here demonstrated that carboxypeptidase S-1 from Penicillium janthinellum (19) exhibits a preference for arginine and lysine in the P_1 position which is most pronounced at pH values above 5.5. It is presumably due to deprotonation of a group on the enzyme with a pK_a around 5.0 promoting the interaction with positively charged substrates. It is furthermore shown that carboxypeptidase S-1, contrary to previous claims (18), exhibits high esterase activity and efficiently catalyses aminolysis reactions.

2. MATERIALS AND METHODS

2.1. Materials

CPD-S-1, partially purified from the growth medium by chromatography on DEAE-Sephadex A-50 and Bio-Gel P-100 (19), was a gift from Dr. THEO HOFMANN, Toronto. FA-Phe-Lys-OH was a gift from Dr. ANDERS KANSTRUP, H.C. Ørsted Institute, Copenhagen. The following materials were prepared as previously described: CABS-Sepharose (5), FA-Phe-Leu-OH (11), Bz-Arg-OBu (7), Bz-Pro-OMe (7), Bz-Gly-OMe (2), Bz-Phe-OMe (2) and Bz-Ile-OMe (7). Bz-Arg-Leu-OH and H-Thr-NH_2 were obtained from Carlsberg Biotechnology, Denmark, and Bz-Val-OMe, Bz-His-OMe and Bz-Met-OMe were from Vega-Fox, USA. H-Val-OH, H-Gly-NH_2 · HCl, H-Arg-OH · HCl, H-Ser-OH, H-Ser-NH_2, Bz-Arg-OMe, Bz-Arg-OEt, Hepes and Mes were from Sigma, USA. All other substrates, amino acids and amino acid derivatives were obtained from Bachem, Switzerland. Sepharose 4B was obtained from Pharmacia, Sweden, and other reagents and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

2.2.1. Purification of CPD-S-1

Carboxypeptidase activity was routinely assayed at 25 °C by following the hydrolysis of FA-Phe-Leu-OH spectrophotometrically at 337 nm using a Perkin-Elmer lambda 7. The following assay mixture was used: 25 µl 8 mM-substrate dissolved in methanol was added to 965 µl 0.05 M-acetic acid, 1 mM-EDTA, pH 4.5, followed by 10 µl enzyme solution. One unit was defined as the amount of enzyme necessary to release one micromole leucine per minute at 25 °C. Protein concentration was determined spectrophotometrically at 280 nm using $A_{280}(1\%) = 26$ (19).

The purification of the crude enzyme, supplied by Dr. THEO HOFMANN, was carried out by means of affinity chromatography: the enzyme was applied to a CABS-Sepharose column, equilibrated with 0.02 M-acetic acid, pH 4.5, at a load of 235 U/ml resin. The resin was washed with the equilibration buffer until A_{280} was below 0.02, and then eluted with 5 mM-benzylsuccinic acid, 0.02 M-acetic acid, pH 4.5. The eluted enzyme was concentrated by ultrafiltration using an Amicon YM 10 membrane until a concentration of approximately 0.5 mg/ml and then dialysed against 0.02 M-acetic acid, pH 4.5 and frozen at -18 °C.

The homogeneity and molecular weight of the isolated enzyme was determined by SDS gel electrophoresis (22). The amino acid composition was determined on a Durrum D-500 amino acid analyser after hydrolysis in 6 M-HCl at 110 °C for 24, 48 and 72 hours. The carbohydrate content was determined by the phenol-sulfuric acid method (17).

2.2.2. Stability of CPD-S-1

The stability of CPD-S-1 at 25 °C and different pH values was determined by incubating the enzyme at a concentration of around 0.1 mg/ml in 20 mM-buffer, 0.2 M-NaCl, 1 mM-EDTA, using the following buffers: Mes, pH 5.5-7.0; Hepes, pH 7.1-8.0; acetic acid, pH 4.3-5.4; formic acid, pH 3.3-4.2. The enzymatic activity towards either FA-Phe-Leu-OH or Bz-Arg-OBu was determined in the buffer used for the incubation. The resistance of the enzyme towards water-miscible organic solvents was tested by adding the enzyme (final concentration: 0.04 mg/ml) to various mixtures of solvent and 0.1 M-Mes, 1 mM-EDTA, pH 6.0 or 0.1 M-formic acid, 1 mM-EDTA, pH 4.0.

2.2.3. Enzymatic properties of CPD-S-1

All enzymatic activities were determined spectrophotometrically at 230 nm for Z-substrates, 254 nm for Bz-substrates and at 329-350 nm for FA-substrates using a Cary 219 or a Perkin-Elmer lambda 7 spectrophotometer, thermostated at 25 °C. k_{cat} and K_m values were graphically determined from Lineweaver-Burk plots. The pH profiles of the kinetic parameters were based on assays in the buffer-system listed in section *2.2.2.* The substrate preference of CPD-S-1 was investigated by determination of k_{cat}/K_m values for the hydrolysis of a series of Z-Ala-X-OH and Bz-X-OMe substrates (X = amino acid residue). 25 µl of a 20 mM-substrate solution in methanol was added to 965 µl 0.05 M-Mes, 1 mM-EDTA, pH 6.2 (for the Bz-substrates) or 965 µl 0.05 M-acetic acid, 1 mM-EDTA, pH 4.1 (for the Z-substrates), followed by the addition of 10 µl enzyme.

Aminolysis reactions (1.5 ml) were carried out in a pH stat (23) in the presence of 5 mM-EDTA. Unless specifically stated the reactions were allowed to proceed until 80-95% of the substrate had been consumed. At this stage aliquots were removed, the reaction terminated by addition of four volumes CH_3CN and the reactant composition determined by HPLC, using equipment from Waters Associates, USA, and a 5 µ Nova-Pak C-18 reverse phase column. The following eluent systems were used: 0.1% trifluoroacetic acid (A-buffer) and 50% CH_3CN, 0.1% tri-fluoroacetic acid (B-buffer) or 50 mM-triethyl ammonium phosphate, pH 3.0 (A-buffer) and 50 mM-triethyl ammonium phosphate, pH 3.0 in 50% CH_3CN (B-buffer). Various linear and concave gradients were employed. All separations were carried out at room temperature and monitored at 254 nm. The composition of the reaction mixture was estimated directly from the integrated peak areas since all components had the Bz-group as dominant chromophore. Aminolysis and hydrolysis products were collected and identified by amino acid analysis. The fraction of aminolysis was expressed as the ratio between the formed aminolysis product and the sum of all products being formed, i.e. unconsumed substrate was disregarded in the calculations.

3. RESULTS AND DISCUSSION

3.1. Purification and physical chemical properties of CPD-S-1

CPD-S-1, previously subjected to ion exchange chromatography and gel filtration (19) was purified by affinity chromatography on CABS-Sepharose in a yield of around 40% using a procedure similar to those utilised for the purification of malt carboxypeptidases I, II and III (5, 12, 15). The specific activity of the purified enzyme towards FA-Phe-Leu-OH was 21 U/mg. SDS gel electrophoresis indicated homogeneity and a molecular weight of 48,000 as previosuly reported (19). The amino acid composition was identical to that previously reported (19), and the content of neutral sugar was found to be 3%.

CPD-S-1 was stable at 25 °C for 4 hours at pH values between pH 3.0 and 6.5, while at pH 7.0 and 8.0 the $t_{1/2}$ values were 6 hours and 45 minutes, respectively. CPD-S-1 was rapidly inactivated at 25 °C in 80% methanol and in 60% DMF while it was essentially stable at both solvent concentrations at 10 °C; at lower concentrations of solvent the enzyme was more stable (see Table I). In comparison with other serine carboxypeptidases, CPD-S-1 exhibits a stability at alkaline pH similar to malt carboxypeptidase I (5) while it is less stable than malt carboxypeptidase II (12) and in particular carboxypeptidase Y. In organic solvents the

Table I. The stability of CPD-S-1 in organic solvents

Solvent (v/v)	Half-life 25 °C	Half-life 10 °C
60% methanol	8½h	
70% methanol	35 min	> 24 h
80% methanol	5 min	5½h
40% DMF	> 24 h	
60% DMF	15 min	7 h
40% 1-propanol	2 h	

The enzyme ($5 \cdot 10^{-7}$ M) was incubated in a mixture of 0.1 M-sodium formate, 1 mM-EDTA pH 4.0 and the indicated solvent. The activity of the enzyme towards FA-Phe-Leu-OH was determined with time.

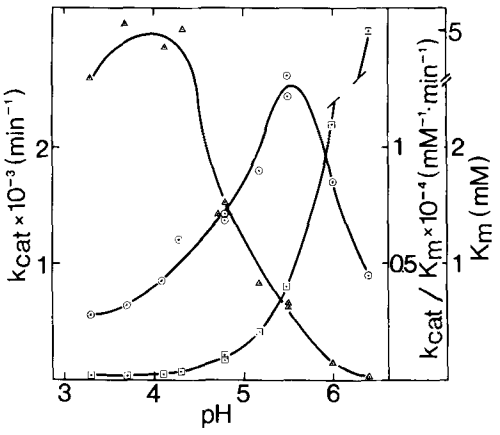

Figure 1. The influence of pH on the kinetic parameters for the CPD-S-1 catalysed hydrolysis of FA-Phe-Leu-OH. The assays were performed in 20 mM-buffer, 0.2 M-NaCl, 1 mM-EDTA, using the buffers listed in section *2.2.2*. -⊙-⊙-, k_{cat}; -⊡-⊡-, K_m; -Δ-Δ-, k_{cat}/K_m.

extracellular CPD-S-1 is much more stable than the other serine carboxypeptidases of intracellular origin (unpublished results).

3.2. Enzymatic properties of CPD-S-1

Serine carboxypeptidases catalyse the release of amino acids from the C-terminus of peptides with a pH optimum in the range 4-5.5 and the release of alcohols and ammonia from peptide esters and amides, respectively, with a somewhat more alkaline pH optimum. The extent to which CPD-S-1 fits into this pattern was investigated by studying the pH dependence for the hydrolysis of the peptide substrate FA-Phe-Leu-OH and the ester substrate FA-Phe-OMe. Using 0.2 mM-substrate and 0.05 M-buffer the hydrolysis of FA-Phe-Leu-OH was optimal at pH 4.0-4.5 with a specific activity of 21 U/mg whereas the esterase activity towards FA-Phe-OMe was optimal at pH 6.0-6.3 with a specific activity of only 0.18 U/mg consistent with the results previously reported by FUKUDA et al. (18). However, this difference in the levels of activities towards corresponding peptide and ester substrates is unusual for serine carboxypeptidases. To understand this difference the influence of pH was assessed by determination of the kinetic parameters for the hydrolysis of FA-Phe-Leu-OH (the low activity towards FA-Phe-OMe did not permit a similar study with this substrate). k_{cat} for the hydrolysis of FA-Phe-Leu-OH increased with pH and reached a maximum at pH 5.5 (Figure 1), apparently dependent on the deprotonation of more than a single group on the enzyme. At pH > 5.5 k_{cat} decreased, K_m increased steeply and the peptidase activity of the enzyme (k_{cat}/K_m) was drastically reduced. With other serine carboxypeptidases deprotonation of a positively charged binding site for the C-terminal carboxylate group of peptide substrates, also causes an increase in K_m and in some cases a decrease in k_{cat} but this takes place at significantly higher pH (pH > 6.5) (14). The decrease of CPD-S-1 catalysed peptide hydrolysis in the pH range 5-6 is probably due to deprotonation of a different group on the enzyme. If this group in addition has an adverse effect on the hydrolysis of substrates with blocked C-terminus it might explain the low activity towards FA-Phe-OMe in the pH range 5-7.

The basis for the widely different levels of activity towards FA-Phe-Leu-OH and FA-Phe-OMe was further investigated by determination of the substrate preference of CPD-S-1. k_{cat}/K_m values were determined for the hydrolysis of a series of Bz-X-OMe ester substrates at pH 6.2 and a series of Z-Ala-Y-OH substrates at pH 4.1 (X and Y = amino acid residues) (Table II). The results indicate that the enzyme, contrary to the

Table II. Hydrolysis of Z-Ala-X-OH and Bz-Y-OMe substrates (X and Y = amino residue) with serine carboxypeptidases

Substrate	k_{cat}/K_m (min^{-1} · mM^{-1})				
	CPD-S-1	CPD-M_I	CPD-M_{II}	CPD-M_{III}	CPD-Y
Z-Ala-Gly-OH	4	480	11	11	< 30
Z-Ala-Ala-OH	390	22000	560	2100	13000
Z-Ala-Val-OH	410	35000	1800	17000	15000
Z-Ala-Ile-OH	340	22000	3300	27000	32000
Z-Ala-Met-OH	1400	18000	3200	43000	56000
Z-Ala-Phe-OH	1600	5700	2100	94000	19000
Z-Ala-Pro-OH	4	2600	6	120	370
Z-Ala-Asp-OH	190	370	71	< 1	< 30
Z-Ala-Asn-OH	230	270	62	< 1	520
Z-Ala-Lys-OH	2900	< 5	25000	7	520
Z-Ala-Arg-OH	3400	< 5	18000	9	2000
Z-Ala-His-OH	140	120	1100		190
Z-Ala-Ser-OH	72	1080	150	5	1700
Bz-Gly-OMe	< 1	< 5	< 5	< 1	3
Bz-Ala-OMe	3	370	19	200	490
Bz-Val-OMe	< 1	22	7	35	180
Bz-Ile-OMe	< 1	17	8	34	260
Bz-Leu-OMe	42	420	310	820	5600
Bz-Met-OMe	150	1200	160	750	2800
Bz-Phe-OMe	49	14600	5700	1000	51000
Bz-Asp-OMe	< 1	< 5	< 5	2	< 1
Bz-Lys-OMe	2700	18300	2200	3	1
Bz-Arg-OMe	10200	25300	950	18	12
Bz-His-OMe	81	5600	76	23	13
Bz-Thr-OMe	< 1	69	< 5	5	18
Bz-Pro-OMe	< 1	< 5	< 5	< 1	< 1

Assay conditions for CPD-S-1: 0.5 mM-substrate, 2.5% CH_3OH, 0.05 M-acetic acid, 1 mM-EDTA, pH 4.1 with Z-substrates and 0.5 mM-substrate, 2.5% CH_3OH, 0.05 M-Mes, 1 mM-EDTA, pH 6.2 with Bz-substrates. With Bz-Lys-OMe, Bz-Arg-OMe, Z-Ala-Arg-OH and Z-Ala-Lys-OH CH_3OH was omitted. The rate of hydrolysis was followed to more than 95% completion. From the progression curves k_{cat}/K_m values were determined utilizing the integrated form of the Michaelis-Menten equation. The values for the other carboxypeptidases were from references 5, 7, 12, 13, 15, 16. With CPD-S-1, CPD-Y and CPD-M_{III} the values were based on the molecular weights of the native enzyme, i.e. 48,000, 64,000 and 48,000, respectively. In the case of the dimeric CPD-M_I and CPD-M_{II} the values were based on the molecular weights of the monomer, i.e. 56,000 and 60,000, respectively.

statement of FUKUDA et al. (18), exhibits high esterase activity but only towards ester substrates with basic amino acid residues in the P_1 position, i.e. X = Arg, Lys. The preference with respect to the P_1' position of peptide substrates is less pronounced: the highest k_{cat}/K_m values were obtained with Y = Arg, Lys, Phe, Met, i.e. the enzyme releases both basic and hydrophobic amino acid residues with high rates.

The preference for basic amino acid residues in the P_1 and P_1' positions was studied in further detail with the peptide substrates FA-Phe-Lys-OH and Bz-Arg-Leu-OH and the ester substrate Bz-Arg-OBu. With FA-Phe-Lys-OH (Figure 2) the pH profiles for k_{cat} and in particular K_m were similar to those obtained with FA-Phe-Leu-OH. Thus, the adverse effects on the peptide hydrolysis in the pH range 5-6 are apparently only to

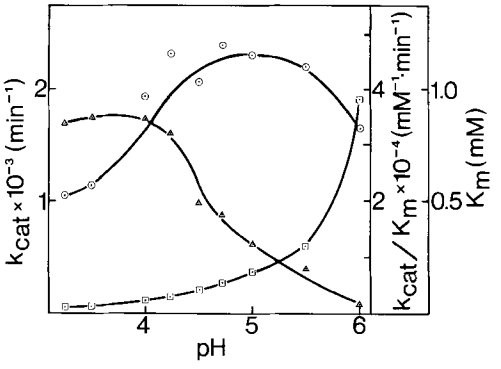

Figure 2. The influence of pH on the kinetic parameters for the CPD-S-1 catalyzed hydrolysis of FA-Phe-Lys-OH. Buffers and symbols as in Figure 1.

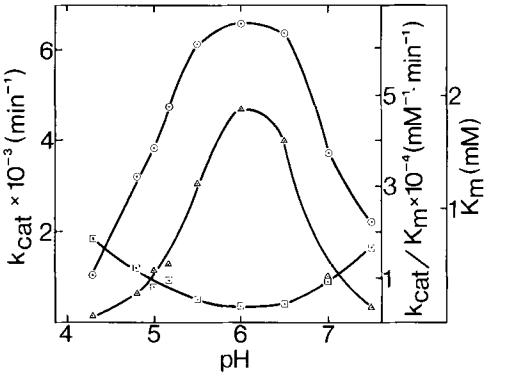

Figure 4. The influence of pH on the kinetic parameters for the CPD-S-1 catalysed hydrolysis of Bz-Arg-OBu. Buffers and symbols as in Figure 1.

a minor extent dependent on the nature of the C-terminal amino acid residue of the peptide substrate. With Bz-Arg-Leu-OH (Figure 3) k_{cat} is dependent on the deprotonation of a group on the enzyme with a pK_a of approximately 5.2 and on the protonation of a group with a pK_a around 6.6, the highest k_{cat} values being observed around pH 6.0. The pH profile for k_{cat} is similar with Bz-Arg-OBu (Figure 4), the corresponding pK_a values being around 5.0 and 7.0. K_m for the hydrolysis of Bz-Arg-Leu-OH is dependent on the deprotonation of a group with a $pK_a > 6.7$, probably the group functioning as the binding site for the C-terminal carboxylate group (see above). K_m for the hydrolysis of Bz-Arg-OBu decreased somewhat in the pH range 4.3-6.0 and increased in the pH range 6.5-7.5, but no assignments with respect to particular groups can be made.

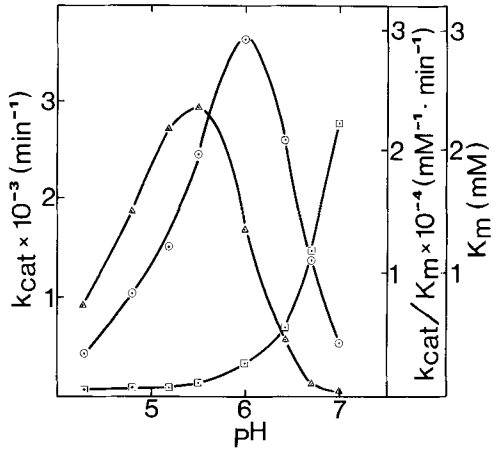

Figure 3. The influence of pH on the kinetic parameters for the CPD-S-1 catalysed hydrolysis of Bz-Arg-Leu-OH. Buffers and symbols as in Figure 1.

Since the hydrolysis of Bz-Arg-OBu and Bz-Arg-Leu-OH is favourably affected and the hydrolysis of FA-Phe-Leu-OH and FA-Phe-Lys-OH is adversely affected in the range of pH 5-6 it is possible that the ionic state of a single group causes these effects. This might be carboxylic acid group which is deprotonated. A negatively charged carboxylate group might bind to the positively charged arginyl residues at the P_1 position of Bz-Arg-OBu and Bz-Arg-Leu-OH while the interaction with the hydrophobic phenylalanyl residues of FA-Phe-Leu-OH and FA-Phe-Lys-OH is weakened. The preference of the enzyme with respect to the P_1 position is therefore pH dependent as evidenced by the different pH optima of k_{cat}/K_m for the hydrolysis of FA-Phe-Leu-OH and Bz-Arg-Leu-OH, i.e. around 4.1 and 5.5, respectively.

The effect of pH on specificity was confirmed by digestion of the B-chain of oxidised porcine insulin with the C-terminal sequence -Cys(SO_3^-) -Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-OH. At pH 6.5, the C-terminal alanyl residue was rapidly released and the digestion was then halted; only prolonged incubation

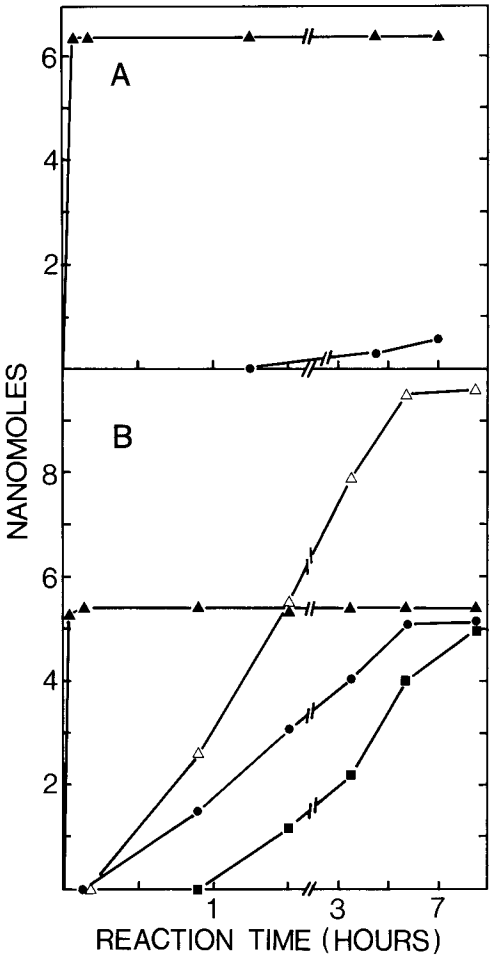

Figure 5. Digestion of the B-chain of oxidised porcine insulin with CPD-S-1. Reaction conditions: 0.3 mM B-chain, $3.8 \cdot 10^{-7}$ M CPD-S-1, 1 mM-EDTA and 20 mM-Mes, pH 6.5 (Panel A) or 0.05 M-acetic acid, 1 mM-EDTA, pH 3.2 (Panel B). At the indicated times 50 µl aliquots were withdrawn from the reaction mixture, acidified by addition of 5 µl 1 M-HCl acid and 20 µl applied directly to the amino acid analyser. ▲, Ala; ●, Lys, Pro, Thr, Tyr; △, Phe; ■, Arg, Gly.

Table III. The influence of NaCl on the kinetic parameters for CPD-S-1 catalysed hydrolysis of ester, peptide and amide substrates

Substrate		[NaCl] 0	[NaCl] 0.2 M
Bz-Arg-OBu[a)]	k_{cat}	6,600	6,800
	K_m	0,083	0,14
	k_{cat}/K_m	80,000	48,000
Bz-Arg-Leu-OH[a)]	k_{cat}	3,600	3,600
	K_m	0,11	0,27
	k_{cat}/K_m	33,000	13,000
FA-Phe-Leu-OH[b)]	k_{cat}	940	1,000
	K_m	0,058	0,071
	k_{cat}/K_m	16,000	14,000
FA-Phe-Lys-OH[d)]	k_{cat}	2,100	2,100
	K_m	0,053	0,106
	k_{cat}/K_m	40,000	20,000
Bz-Arg-NH_2[a,c)]	k_{cat}/K_m	730	240

Assay conditions:
a) 0.02 M-Mes, 1 mM-EDTA, pH 6.0
b) 0.05 M-sodium acetate, 1 mM-EDTA, 2.5% CH_3OH, pH 4.1
c) The high K_m value (>5 mM) prevented determination of the k_{cat} and K_m values. k_{cat}/K_m was determined utilizing the integrated form of the Michaelis-Menten equation (see text for Table II)
d) 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5

resulted in release of the subsequent amino acids (Figure 5A). Thus, the Lys-Ala bond is cleaved very rapidly and the following Pro-Lys bond is cleaved with negligible rate, consistent with the positively charged lysyl residue being much more preferred at the P_1 position at pH 6.5 than the hydrophobic prolyl residue. At pH 3.2, the Pro-Lys bond is cleaved at significant rate consistent with protonation of a group on the enzyme influencing its specificity (Figure 5B). Lys and the following five amino acids, Pro, Thr, Tyr and two Phe, are released with identical rates (as a "block") and then Arg and Gly as another block. It thus appears that the cleavage of the Pro-Lys and Arg-Gly are rate-limiting for the digestion.

The importance of ionic interactions for the hydrolysis of substrates with basic amino acid residues in the P_1 position is supported by results listed in Table III. k_{cat}/K_m for the hydrolysis of Bz-Arg-Leu-OH, FA-Phe-Lys-OH, Bz-Arg-OBu and Bz-Arg-NH_2 is reduced by addition of 0.2 M-NaCl, primarily due to increased K_m values, and hence, NaCl acts as a competitive inhibitor. In comparison, k_{cat}/K_m for the hydrolysis of FA-Phe-Leu-OH is only slightly affected by increased ionic strength.

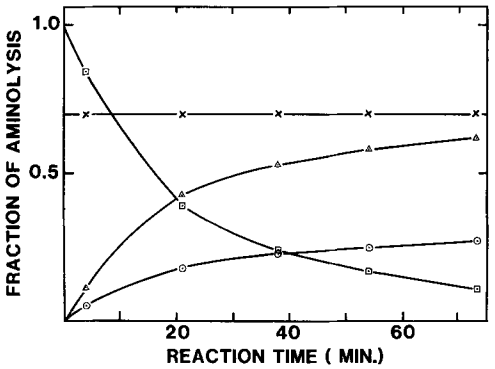

Figure 6. CPD-S-1 catalysed aminolysis of Bz-Arg-OBu using H-Arg-OH as nucleophile. Reaction conditions: 2 mM-Bz-Arg-OBu, 1 M-H-Arg-OH, 5 mM-EDTA, $9.9 \cdot 10^{-8}$ M-CPD-S-1, pH 7.0. Reactions were terminated at the indicated times and analysed by HPLC (see section 2.2). -□-□-, Bz-Arg-OBu; -⊙-⊙-, Bz-Arg-OH; -△-△-, Bz-Arg-Arg-OH; -×-×-, fraction of aminolysis, i.e. Bz-Arg-Arg-OH/(Bz-Arg-OH + Bz-Arg-Arg-OH).

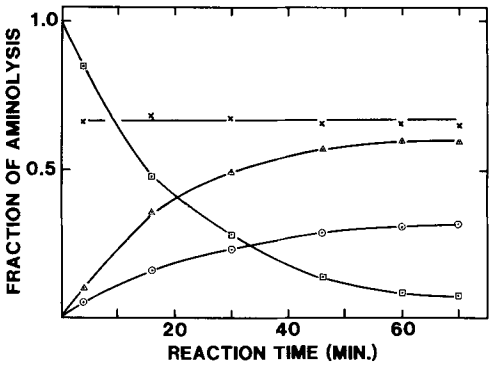

Figure 7. CPD-S-1 catalysed aminolysis of Bz-Arg-Arg-OH using H-Arg-NH_2 as nucleophile. Reaction conditions: 2 mM-Bz-Arg-Arg-OH, 1 M-H-Arg-NH_2, 5 mM-EDTA, $1.2 \cdot 10^{-7}$ CPD-S-1, pH 6.0. Reactions were terminated at the indicated times and analysed by HPLC (see section 2.2). -□-□-, Bz-Arg-Arg-OH; -⊙-⊙-, Bz-Arg-OH; -△-△-, Bz-Arg-Arg-NH_2; -×-×-, fraction of aminolysis, i.e. Bz-Arg-Arg-NH_2/(Bz-Arg-OH + Bz-Arg-Arg-NH_2).

3.3. CPD-S-1 catalysed peptide synthesis

The ability of CPD-S-1 to catalyse aminolysis reactions was studied at pH <7.5 due to the instability of the enzyme at higher pH values. Like other serine carboxypeptidases (14) CPD-S-1 catalyses the aminolysis of N-blocked amino acid esters and N-blocked dipeptides (transpeptidation reactions) using amino acids and amino acid amides as nucleophiles. However, unlike other serine carboxypeptidases (10, 13, 24) CPD-S-1 does not accept amino acid methyl esters as nucleophiles, i.e. H-Gly-OMe and H-Val-OMe. CPD-S-1 hydrolyses ester and amide substrates with more alkaline pH optima than it hydrolyses peptide substrates. Thus, when aminolysis of ester substrates, using amino acids as nucleophiles, is performed at a sufficiently high pH, degradation of the produced peptide is reduced to a minimum. When the reaction with Bz-Arg-OBu as substrate and H-Arg-OH as nucleophile was carried out at pH 6.0 the fraction of aminolysis decreased during the course of the reaction, indicating that the product (Bz-Arg-Arg-OH) was degraded during the reaction. At pH 7.0 the fraction of aminolysis remained constant at 0.70, independent of the extent of the reaction (Figure 6). With Bz-Arg-OBu as substrate and H-Arg-NH_2 as nucleophile the fraction of aminolysis remained constant both at pH 6.0 and pH 7.0 (data not shown), consistent with the activity of the enzyme towards the peptide amide product (Bz-Arg-Arg-NH_2) being much lower than that towards the ester substrate, regardless of pH (data not shown).

The influence of pH on the transpeptidation reaction with Bz-Arg-Leu-OH as substrate and H-Arg-NH_2 as nucleophile was studied in a similar way. At pH 7.0 the fraction of aminolysis decreased with the extent of the reaction, suggesting that the product, Bz-Arg-Arg-NH_2, was degraded by the enzyme. However, at pH 6.0 the fraction of aminolysis remained constant around 0.67 (Figure 7), consistent with the effect of pH on the amidase and peptidase activities of CPD-S-1.

The influence of the concentration of the nucleophile on the fraction of aminolysis was studied with Bz-Arg-OBu as substrate and H-Val-OH and H-Val-NH_2 as nucleophiles (Figure 8). With H-Val-NH_2 the fraction of aminolysis, i.e. formation of Bz-Arg-Val-NH_2, reached a constant value at 0.95, suggesting that the nucleophile binds to the acyl-enzyme intermediate

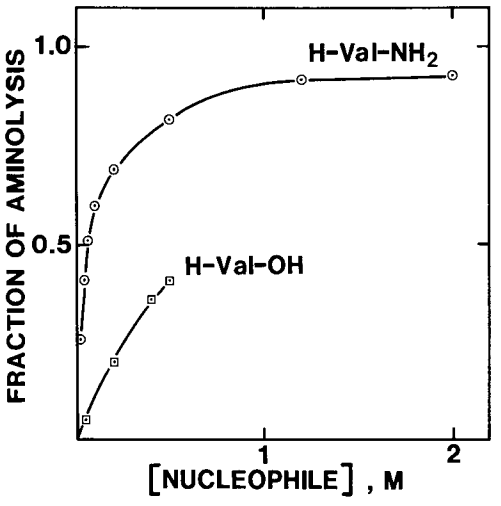

Figure 8. The influence of the nucleophile concentration on the fraction of aminolysis using Bz-Arg-OBu as substrate and H-Val-NH_2 and H-Val-OH as nucleophiles. The reaction conditions were: 2 mM-Bz-Arg-OBu, 5 mM-EDTA, $2.5 \cdot 10^{-8}$-10^{-7} M-CPD-S-1, pH 7.0. The concentration of nucleophile is indicated. Reaction time: 5-20 minutes. 80-95% of the substrate was consumed.

prior to the deacylation reaction (10). The concentration of nucleophile where the fraction of aminolysis is half the maximal value, which is designated $K_{N(app)}$ and is a measure for the dissociation constant of the complex between acyl-enzyme intermediate and nucleophile (10), is 0.06 M. With H-Val-OH the reaction could only be studied at concentrations below 0.5 M due to the limited solubility of this nucleophile, and at this concentration the fraction of aminolysis had not reached a constant level. Thus, it cannot be established whether this amino acid binds to the acyl-enzyme intermediate, but if it does the $K_{N(app)}$ exceeds 0.3 M. In any event, it can be concluded that the carboxyamide group of H-Val-NH_2 cause better binding of the nucleophile to the enzyme than the negatively charged carboxylate group of H-Val-OH.

The influence of the type of substrate utilised in the aminolysis reaction on $K_{N(app)}$ and the maximum level of aminolysis was systematically studied with Bz-Arg-OBu and Bz-Arg-Leu-OH, two substrates which are of similar size and produce the same acyl-enzyme intermediate but differ with respect to the nature of the scissile bond and the charge of the leaving group (Table IV). Using the nucleophiles H-Gly-NH_2, H-Val-NH_2 and H-Arg-NH_2 the $K_{N(app)}$ was slightly lower and the fraction of aminolysis at saturation slightly higher with the ester substrate as compared with the peptide substrate. To test whether the size and/or hydrophobicity of the C-terminal amino acid residue of peptide substrates had an influence, the aminolysis reaction using H-Gly-NH_2 was carried out with Bz-Arg-Ser-OH as well. It was found that neither $K_{N(app)}$ nor the maximum fraction of aminolysis deviated significantly from the values obtained with

Table IV. CPD-S-1 catalysed synthesis of peptide bonds: the influence of the substrate leaving group

	Nucleophile	Aminolysis product	Frac. of aminolysis at saturat.	$K_{N(app)}$ (M)
Bz-Arg-OBu	H-Gly-NH_2	Bz-Arg-Gly-NH_2	0.85	0.13
Bz-Arg-Leu-OH	H-Gly-NH_2	Bz-Arg-Gly-NH_2	0.75	0.17
Bz-Arg-Ser-OH	H-Gly-NH_2	Bz-Arg-Gly-NH_2	0.85	0.14
Bz-Arg-OBu	H-Val-NH_2	Bz-Arg-Val-NH_2	0.70	0.25
Bz-Arg-Leu-OH	H-Val-NH_2	Bz-Arg-Val-NH_2	0.65	0.30
Bz-Arg-OBu	H-Arg-NH_2	Bz-Arg-Arg-NH_2	0.80	0.007
Bz-Arg-Leu-OH	H-Arg-NH_2	Bz-Arg-Arg-NH_2	0.70	0.02

The following conditions were used: 2 mM-substrate, 0.005-2 M-nucleophile, 5 mM-EDTA, pH 6.0. The concentration of CPD-S-1 varied from $6 \cdot 10^{-8}$-$2 \cdot 10^{-7}$ M and the reaction time was 5-30 minutes.

Bz-Arg-Leu-OH. It is thus appararent that the deacylation reaction is essentially independent of the nature of the leaving group, suggesting that dissociation of the leaving group from the enzyme is not rate limiting in CPD-S-1 catalysed aminolysis reactions. In carboxypeptidase Y catalysed transpeptidation reactions, on the other hand, high fractions of aminolysis were obtained only when the the C-terminal position of the peptide substrate was occupied by a small and/or hydrophilic amino acid residue (1, 3), suggesting that released hydrophobic amino acids prevent productive binding of the added nucleophile without impeding the attack of water on the acyl-enzyme intermediate.

The side-chain specificity of CPD-S-1 in hydrolysis reactions (Table II), combined with the ability of the enzyme to catalyse aminolysis reactions with amino acids and amino acid amides as nucleophiles suggest that it is a useful tool for synthesis of -Arg-X- and -Lys-X- peptide bonds (X = amino acid residue). The ability of the enzyme to elongate peptide chains was studied with Bz-Arg-OBu as substrate and a series of amino acids and amino acid amides as nucleophiles at pH 7.0 where the products, Bz-Arg-X-OH or Bz-Arg-X-NH_2, accumulate in the reaction mixture since they are degraded with negligible rates. To maximise the fraction of aminolysis amino acids were utilised at a concentration only slightly less than the maximum obtainable. Amino acid amides, on the other hand, bind better to the enzyme (see Figure 6) such that maximum aminolysis in most cases could be achieved at much lower concentrations.

With free amino acids high fractions of aminolysis (above 0.60) were obtained with H-Gly-OH, H-Ala-OH, H-Ser-OH, H-Arg-OH, H-Lys-OH and H-His-OH (Table V), medium values (0.3-0.5) were obtained with H-Thr-OH, H-Val-OH, H-Phe-OH, H-Glu-OH and H-Gln-OH, and low values (0-0.25) with H-Leu-OH, H-Pro-OH and H-Asp-OH. With all the corresponding amino acid amides, with the exception of H-Pro-NH_2, H-Glu-α-NH_2 and H-Asp-α-NH_2, the fraction of aminolysis were high. Compared to aminolysis reactions catalysed by

Table V. CPD-S-1 catalyzed aminolysis reactions using Bz-Arg-OBu as substrate and amino acids and amino acid amides as nucleophiles

Amino acids (conc.)	fractions of aminolysis	Amino acid amides (conc.)		fraction of aminolysis
H-Gly-OH (3 M)	0.90	H-Gly-NH_2	(2 M)	0.95
H-Ala-OH (1.5 M)	0.60	H-Ala-NH_2	(2 M)	0.95
H-Ser-OH (3 M)	0.60	H-Ser-NH_2	(2 M)	0.95
H-Thr-OH (0.9 M)	0.45	H-Thr-NH_2	(2 M)	-
H-Val-OH (0.5 M)	0.40	H-Val-NH_2	(1.5 M)	0.95
H-Leu-OH (0.15 M)	0.10	H-Leu-NH_2	(1 M)	0.60
H-Met-OH (0.4 M)	0.60	H-Met-NH_2	(1 M)	0.95
H-Phe-OH (0.15 M)	0.35	H-Phe-NH_2	(0.5 M)	0.85
H-Pro-OH (5 M)	0	H-Pro-NH_2	(1 M)	0
H-Glu-OH (1 M)	0.35	H-Glu-α-NH_2	(1 M)	0.20
H-Gln-OH (0.45 M)	0.40	H-Gln-NH_2	(1 M)	0.90
H-Asp-OH (1.1 M)	0.15	H-Asp-α-NH_2	(0.5 M)	0.15
H-Asn-OH (0.17 M)	0.05	H-Asn-NH_2	(1 M)	0.90
H-Arg-OH (1 M)	0.70	H-Arg-NH_2	(0.1 M)	0.70
H-Lys-OH (1.5 M)	0.70	H-Lys-NH_2	(0.5 M)	0.95
H-His-OH (0.65 M)	0.70	H-His-NH_2	(0.5 M)	0.95

The reactions were performed with 2 mM-Bz-Arg-OBu, 1 mM-EDTA and the indicated concentration of nucleophile at pH 7.0. The enzyme concentrations varied between $3 \cdot 10^{-8}$ M and $3 \cdot 10^{-7}$ M and the reaction time was 5-60 minutes.

Table VI. CPD-S-1 catalyzed transpeptidation reactions using Bz-Arg-Leu-OH as substrate and amino acid amides (X) as nucleophiles

Nucleophile (conc.)	Fraction of aminolysis (Bz-Arg-X-NH_2)
H-Gly-NH_2 (2 M)	0.75
H-Ala-NH_2 (2 M)	0.70
H-Thr-NH_2 (2 M)	0.85
H-Val-NH_2 (1.5 M)	0.60
H-Met-NH_2 (1 M)	0.60
H-Phe-NH_2 (0.5 M)	0.50
H-Arg-NH_2 (1 M)	0.70
H-His-NH_2 (1 M)	0.60

The reactions were performed with 2 mM-Bz-Arg-Leu-OH, 1 mM-EDTA and the indicated concentrations of nucleophile at pH 6.0. The enzyme concentrations varied between $6 \cdot 10^{-8} - 2 \cdot 10^{-7}$ M and the reaction time was 5-60 minutes.

carboxypeptidase Y, which is the only other serine carboxypeptidase which has been investigated in similar detail, the fractions of aminolysis were generally of the same order of magnitude with the exception of the reactions with H-Gly-OH, H-Glu-OH, H-Gln-OH, H-Arg-OH, H-Lys-OH and H-His-OH where the fractions of aminolysis with CPD-S-1 were significantly higher than with carboxypeptidase Y.

Transpeptidation reactions were carried out with Bz-Arg-Leu-OH as substrate and various amino acid amides as nucleophiles, thus producing peptide amides of the general formula Bz-Arg-X-NH_2 (X = amino acid residue) (Table VI). It was found that the fraction of aminolysis varied between 0.50 and 0.85, and thus, it appears that CPD-S-1 efficiently catalyses the incorporation of most C-terminal amino acid residues. However, the highest reaction rates are obtained when the substrate contains Lys or Arg at the P_1 position (see Table II).

The high stability of CPD-S-1 in methanol warranted an investigation of its ability to catalyse the conversion of peptides to peptide esters. The reactions were carried out at acidic pH where the activity towards peptides is maximal and that towards esters minimal and hence, the degradation of the ester product minimal. The conversion of Bz-Arg-Leu-OH to Bz-Arg-OMe in 80% methanol at an apparent pH of 4.0 and 10 °C proceeded only with an extremely low rate and a fraction of alcoholysis around 0.3 (Table VII). A similar result was obtained with FA-Ala-Lys-OH in 50% methanol. Carboxypeptidase Y was only tested at concentrations below 40% methanol due to its lack of stability at higher concentrations. However, the fractions of alcoholysis were of the same order of magnitude.

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Table VII. CPD-S-1 catalysed esterifications utilising methanol as nucleophile

Substrate	Composition of reaction mixture				
	Reaction time	R-X-Y-OH (%)	R-X-OH (%)	R-X-OMe (%)	Fraction of alcoholysis
Bz-Arg-Leu-OH[a)]	23 h	5	68	27	0.28
FA-Ala-Lys-OH[b)]	18 h	25	51	24	0.32

Reaction conditions: [a)] 800 µl CH_3OH + 150 µl 0.2 M-sodium formate, 5 mM-EDTA, pH 4.0 + 50 µl CPD-S-1 (0.35 mg/ml), 10 mM-substrate, 10 °C, [b)] 500 µl CH_3OH + 490 µl 0.2 M-sodium formate, 5 mM-EDTA, pH 4.0 + 10 µl CPD-S-1 (0.35 mg/ml), 10 mM-substrate, 10 °C.

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