SPECIFIC WATER-SOLUBLE SUBSTRATES FOR CHYMOTRYPSIN: ATTEMPTS FOR COMPENSATING DIMINISHED P_1–S_1 INTERACTIONS

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Dedicated to the memory of Dr Karel Bláha.

N-Maleyl-L-amino acid and peptide esters were synthesized and employed as substrates for α-chymotrypsin. From the k_{cat}/K_M values can be suggested that benzyl esters are significantly better substrates than the appropriate methyl esters. Further improvement in the substrate properties results from the introduction of the *p*-nitrobenzyl ester moiety. The choline ester of benzyloxycarbonyl-L-phenylalanine with the highest k_{cat}/K_M value confirmed the P_1' leaving group specificity for positively charged residues. From the kinetic data can be concluded that acyl donors with high k_{cat}/K_M values, which are useful in kinetically controlled enzymatic peptide synthesis, need not contain aromatic amino acid residues in the P_1 position.

It is known from X-ray crystallographic studies[1,2] that α-chymotrypsin* has a well-defined binding pocket for the aromatic side chain of the amino acid residue in P_1 position and a hydrogen bonding site for the NH of the acylamino group (binding site notation according to Schechter and Berger[4]). The peptide derived acyl portion of the substrate binds in a specificity pocket with antiparallel β-type configuration with residues 214–216 of the enzyme[5]. If either of these anchors is missing, the reaction rate drops dramatically. The relative narrow substrate specificity of α-chymotrypsin is a limitation for its use in enzymatic peptide synthesis[6–8]. To circumvent this limitation we have studied the chymotryptic hydrolysis of ester substrates differing both in the P_1 amino acid residue and the kind of ester moiety. With the exception of the choline ester the maleyl protecting group has been used, especially due to its solubility-promoting properties. The structural requirements for effective ester substrates have been determined by means of steady state kinetics.

* Symbols and nomenclature follow the recommendations published by IUPAC-IUB Commision on Biochemical Nomenclature for amino acids[3]. The chiral amino acids appearing in this paper are of the L series. Further abbreviations: Cb-, 3-carboxybenzoyl; Mal-, maleyl (3-carboxyacryloyl); -OMe, methyl ester; -OBzl, benzyl ester; -ONb, 4-nitrobenzyl ester; -OCho, choline ester.

RESULTS AND DISCUSSION

Using standard procedures methyl, benzyl, and *p*-nitrobenzyl esters of amino acids were synthesized. Direct acylation with maleic anhydride provided the maleyl-amino acid esters. Maleylpeptide esters were synthesized from the appropriate Boc-protected peptide derivatives by removal of the Boc-groups and subsequent acylation with maleic anhydride.

Data of the new substrates for chymotrypsin are listed in Table I. The inherently high water solubility of the ester substrates results from the maleyl group[9]. According to studies of Butler et al.[9] and Rees and Offord[10] the N-maleyl group is removable in aqueous solution or suspension at pH 3·5 with half-times of 11 and 14 h, respectively. The reported remarkable acid lability is based on intramolecular catalysis and is a prerequisite for the application of maleyl-protected derivatives for enzymatic peptide synthesis on a preparative scale.

The kinetic parameters for α-chymotrypsin-catalyzed hydrolysis of the substrates are compiled in Table II. From the series of esters of maleylleucine it can be deduced that benzyl esters are significantly better substrates compared with the related methyl esters. This finding is in accordance with the reported specificity of α-chymo-

TABLE I
Physical data of substrates

Substrate	M.p. °C	$[\alpha]_D^{25}$ (*c* 1, DMF)	R_F System A[a]	R_F system B[a]
Mal-Phe-OMe	82—84	−9·3	0·44	0·92
Mal-Phe-ONb	108—109	−14·7	0·45	0·98
Cb-Phe-OMe	oil	−69·6	0·44	0·96
Mal-Ala-Phe-OMe	157—158	−49·1	0·37	0·84
Mal-Trp-OMe	75—76	+12·9	0·32	0·91
Mal-Tyr-OMe	88—90	+46·8	0·27	0·92
Mal-Leu-OMe	98—99	−46·9	0·41	0·93
Mal-Leu-OBzl	105—106	−43·2	0·45	0·98
Mal-Leu-ONb	103—104	−38·0	0·44	0·96
Mal-Ala-Leu-ONb	163—165	−46·8	0·38	0·92
Mal-Ala_2-Leu-ONb	96—99	−46·7	0·27	0·90
Mal-Leu-Leu-ONb	oil	−51·7	0·49	0·96
Mal-Ala-Ala-ONb	114—115	−10·7	0·35	0·87
Mal-His-ONb	118—119	−16·9	0	0·57
Mal-Met-ONb	oil	−21·8	0·43	0·93
Mal-Ser-ONb	84—86	−22·9	0·27	0·82
Mal-Asp(OBzl)-OBzl	91—92	−13·6	0·51	0·96

[a] Thin layer chromatography was performed on silica gel plates (Silufol, Kavalier); system A: chloroform–methanol (9 : 1); system B: acetic acid–ethyl acetate–water–1-butanol (1 : 1 : 1 : 1)

trypsin towards substrates with hydrophobic leaving groups[11,12]. Further improvement results from the introduction of the *p*-nitrobenzyl ester which may be based on electronic and steric effects of the nitro group. For specific anilide substrates it was reported[13] that substitution in the leaving group may cause steric hindrance in the S_1' subsite of α-chymotrypsin. Similar effects should be discussed for *p*-nitrobenzyl esters.

In the case of the maleylleucine ester series K_M decreases as the hydrophobicity of the leaving group increases, whereas k_{cat} remains nearly constant. Similarly, we found that the *p*-nitrobenzyl ester of maleylphenylalanine causes a significant decrease of K_M compared with the appropriate methyl ester. This behavior can be explained by a rate limiting deacylation of the acyl-enzyme which is essentially the same within these substrate series.

In the series of Mal-, Mal-Ala-, and Mal-Ala-Ala-Leu-ONb k_{cat} increases slightly with decreasing k_M values leading to more specific substrates for chymotrypsin. The increasing k_{cat}/K_M values can be referred to the binding energies of the S_2 and S_3 subsites of α-chymotrypsin[5].

TABLE II

Kinetic constants for α-chymotrypsin-catalyzed hydrolysis of acyl donors

Substrate	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M $mM^{-1}\,s^{-1}$
Mal-Phe-OMe	99 ± 6	13 ± 2	$7{\cdot}6 \cdot 10^3$
Mal-Phe-ONb	105 ± 7	0·30 ± 0·03	$3{\cdot}4 \cdot 10^5$
Cb-Phe-OMe	8·6 ± 0·4	2·4 ± 0·4	$3{\cdot}6 \cdot 10^3$
Mal-Ala-Phe-OMe	65 ± 2	2·4 ± 0·5	$2{\cdot}8 \cdot 10^4$
Z-Phe-OCho	123 ± 2	0·10 ± 0·02	$1{\cdot}2 \cdot 10^6$
Mal-Trp-OMe	61 ± 1	0·95 ± 0·11	$6{\cdot}5 \cdot 10^4$
Mal-Tyr-OMe	127 ± 8	5·8 ± 1·2	$2{\cdot}2 \cdot 10^4$
Mal-Leu-OMe	6·6 ± 0·8	31 ± 2	$2{\cdot}1 \cdot 10^2$
Mal-Leu-OBzl	6·9 ± 0·2	0·73 ± 0·02	$9{\cdot}6 \cdot 10^3$
Mal-Leu-ONb	6·7 ± 0·3	0·20 ± 0·01	$3{\cdot}4 \cdot 10^4$
Mal-Ala-Leu-ONb	8·6 ± 0·2	0·026 ± 0·003	$3{\cdot}3 \cdot 10^5$
Mal-Ala_2-Leu-ONb	9·2 ± 1·3	0·009 ± 0·002	$1{\cdot}0 \cdot 10^6$
Mal-Ala-Ala-ONb	2·7 ± 0·2	0·65 ± 0·08	$4{\cdot}2 \cdot 10^3$
Mal-Leu-Leu-ONb	2·9 ± 0·2	0·0032 ± 0·0004	$9{\cdot}2 \cdot 10^5$
Mal-His-ONb	9·8 ± 0·3	0·41 ± 0·02	$2{\cdot}4 \cdot 10^4$
Mal-Met-ONb	27·3 ± 0·4	0·15 ± 0·01	$1{\cdot}8 \cdot 10^5$
Mal-Ser-ONb	0·84 ± 0·01	2·0 ± 0·1	$4{\cdot}2 \cdot 10^2$
Mal-Asp(OBzl)-OBzl	0·84 ± 0·02	0·85 ± 0·08	$1{\cdot}0 \cdot 10^3$

Comparing the series of Mal-Xaa-ONb the phenylalanine derivative shows the expected high specificity followed by methionine and leucine. Only small differences are seen between leucine and histidine. The imidazole group is uncharged at pH 9·0. It resembles sterically to the phenylalanine side chain but its more hydrophilic properties lead to less favourable S_1–P_1 interactions.

Although Mal-Asp(OBzl)-OBzl has a hydrophobic side chain in P_1 position the substrate properties can not be compared with the other benzyl ester derivatives. The main reason should be the large distance between the aromatic ring and the peptide backbone. A similar effect was observed for methyl esters of ω-phenyl--α-acetamidocarbonic acids[14]. Despite this limitation the aspartic acid derivative is very interesting for preparative α-chymotrypsin-catalyzed synthesis. The excellent substrate properties of the choline ester can be explained both by the P_1' specificity of chymotrypsin for positively charged residues[15] and the Coulomb effect of the positive charge leading to an enhanced reactivity of the choline ester bond[16].

Strict substrate specificity is not always a requirement for kinetically controlled peptide synthesis, because the rate of synthesis is much higher than that of secondary hydrolysis. For this reason, the use of acyl donors with high k_{cat}/K_M values will prevent secondary hydrolysis of the formed peptide product by the same enzyme. The results of our studies indicate that the application of α-chymotrypsin as a biocatalyst for peptide coupling reactions can be extended independently of the rather narrow substrate specificity known from hydrolysis studies. Such a strategy requires acyl donors with suitable leaving groups and if necessary N-terminal prolongated substrates bearing solubilizing blocking groups. In addition, the coupling reaction should be kinetically irreversible in the reaction time frame, since the P_1 amino acid residue does not meet the requirement of hydrolysis specificity.

EXPERIMENTAL

α-Chymotrypsin from bovine pancreas was purchased from Boehringer (three times crystallized, lyophilized, research grade, 47 U/mg) and was used without further purification. Derivatives for the synthesis of the substrates were from Reanal or Reachim. Other chemicals and solvents were reagent grade. Melting points were determined on a Kofler block. The $[\alpha]_D^{25}$ values were estimated on a Polamat A (Zeiss, Jena).

Synthesis of Substrates

Maleylamino acid esters were prepared as described previously[15]. tert-Butyloxycarbonyl--protected peptide *p*-nitrobenzyl esters were prepared by standard procedures[17]. tert-Butyloxycarbonyl groups were removed by treatment with trifluoroacetic acid at room temperature. Maleylpeptide *p*-nitrobenzyl esters were synthesized similarly as described[15] by reaction of peptide *p*-nitrobenzyl ester trifluoroacetate (3 mmol), maleic anhydride (3 mmol) and N-ethylmorpholine (6 mmol) in 10 ml ethyl acetate. The organic phase was washed with 10% $KHSO_4$ solution and water, dried and the product precipitated by addition of diethyl ether. The properties of the substrates are given in Table I. The purity of all substrates was proved by thin layer

chromatography in different solvent systems. The degree of chymotryptic hydrolysis of all substrates was greater than 90%.

Benzyloxycarbonylphenylalanine Choline Ester Iodide

Benzyloxycarbonylphenylalanine(2-dimethylamino)ethyl ester was prepared by coupling benzyloxycarbonylphenylalanine (10 mmol) and 2-dimethylaminoethanol (9 mmol) in 50 ml ethyl acetate using dicyclohexylcarbodiimide (10 mmol). After 30 min the dicyclohexylurea was filtered and the product precipitated by addition of ether. This product was dissolved in 10 ml methyl iodide without further characterization. After 2 days the excess of methyl iodide was evaporated. The resulting benzyloxycarbonylphenylalanine choline ester iodide was recrystallized from methanol–ether. m.p. 136–137°C; R_F 0 (A) 0·57 (B); $[\alpha]_D^{25}$ −26·4 (*c* 1, DMF). For $C_{22}H_{29}N_2O_4I$ (512·4) calculated 51·57% C, 5·70% H, 5·47% N; found 51·76% C, 5·88% H, 5·34% N.

Kinetics of Hydrolysis

Hydrolysis was followed using a Radiometer pH-stat apparatus consisting of a PHM 82. TTT 80, ABU 80 and REC 80. Measurements were made in a reaction vessel thermostatted at 25°C under nitrogen atmosphere at pH 9·0. The reactions were performed in 5 ml 0·2M sodium chloride. Substrate concentrations were in the range of $0{\cdot}2K_M$ to $3K_M$, enzyme concentrations between 0·5 and 500 nM. The kinetic constants and standard deviations were extracted from initial velocities using a nonlinear regression program. Operational normality of α-chymotrypsin was determined by active site titration using *p*-nitrophenylacetate[18]. The enzyme stock solutions were used not longer than two days. Stock solutions of 150 mM substrates, $I = 0{\cdot}2$ (NaCl), pH 7·0, were prepared in the titrator as described previously[19]. The exact concentrations of substrate solutions were determined from alkali uptake at total enzymic hydrolysis.

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