Tetrahedron Vol. 48, No. 6, pp. 1115-1122, 1992
Printed in Great Britain

0040-4020/92 $3.00+.00

Enzymic Synthesis Design and Enzymic Synthesis of Aspartame

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(*Received in UK 26 November* 1991)

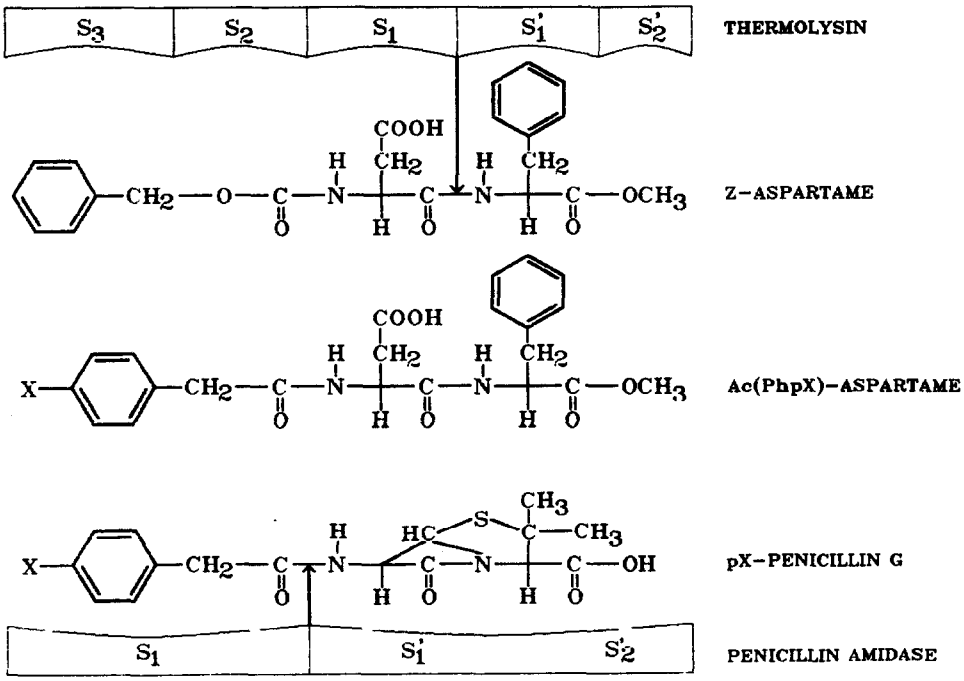
ABSTRACT: *An enzymic synthesis of aspartame (H-Asp-Phe-OMe) has been designed and realized based on the structure-activity study of thermolysin and penicillin amidase hydrolysis of its p-substituted phenylacetyl derivatives. These compounds meet the structural and energetic requirements of two enzymic binding sites The peptide sweetener has been prepared by thermolysin - catalyzed condensation of the p-substituted phenylacetyl-Asp-OH and H-Phe-OMe followed by penicillin amidase - catalyzed deprotection of the resulted aspartame precursors.*

Modern peptide/protein engineering includes three integral consecutive steps: design,synthesis design and synthesis of the peptide/protein. Along with genetic engineering, enzymic synthesis of the peptide bonds affords chirally and chemically pure products[1,2]. It requires a rational enzymic synthesis design, based on structure-reactivity relationships[3].

The industrial production of the low calorie peptide sweetener aspartame (L-aspartyl-L-phenylalanin methyl ester) involves only chemical steps with their inherent shortcomings[4].In an alternative chemical-enzymic synthesis, the enzymic steps are limited either to condensation[5] or deprotection[6]. The combination of the two steps is possible provided the aspartame precursor is a substrate of both the condensation (thermolysin) and the deprotection (penicillin amidase) enzymes. Such a crossreactive

substrate must meet the structural and energetic requirements of the two enzymic binding sites.

The dominant features of the thermolysin specificity can be demonstrated by its interaction with benzyloxycarbonyl (Z) - aspartame (Scheme 1). Being primarily specific to hydrophobic P_1'-amino acids, this neutral proteinase interacts favourably also with hydrophobic P_2- and P_3-amino acid residues accounting for its effectiveness in the synthesis of Z-aspartame[5,7,8]. On the other hand, the penicillin amidase S_1- subsite is specific for the phenylacetyl group Ac(Ph), and the leaving group subsites S_1' and S_2' tolerate structures varying from 6-amino penicillanic acid to aspartame[6,9-11] (Scheme 1). Our preliminary experiments have shown that Ac(Ph)-Asp-OH is acceptable in the thermolysin condensation step, but the yield of Ac(Ph) - aspartame is too low. Since the Z group is of greater length than the Ac(Ph) group, we tentatively supposed that an extension of the latter by hydrophobic p-substituents X would switch on the thermolysin S_3 -subsite in catalysis. Moreover,in the early reports of Cole[9] there are indications that small size p-substituents in Ac(Ph) moiety are acceptable by the penicillin amidase S_1 -subsite. Thus Ac(PhpX)-aspartames would be crossreactive substrates of thermolysin and penicillin amidase.



SCHEME 1.

The effect of p-substitution of Ac(Ph) group by X=-CH_3,-OCH_3 and -NO_2 on the kinetic parameters of thermolysin and penicillin amidase catalyzed hydrolysis of Ac(PhpX)-aspartames is shown in Table 1. The reactivity of these derivatives of Ac(Ph)-aspartame decreases both in thermolysin hydrolysis of the Asp-Phe bond and penicillin amidase hydrolysis of the Ac(PhpX)-Asp bond (k_{cat}/K_m-values decrease).The influence of the p-substituents on the substrate reactivity in the enzyme-catalyzed

Table 1. Kinetic Parameters for Thermolysin and Penicillin Amidase Catalyzed Hydrolysis of Ac(PhpX)-Asp-Phe-OMe[12] and Their Natural Substrates[a]

pX	Thermolysin[b]			Penicillin amidase[c]		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}.mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}.mM^{-1}$)
-H	0.81	0.35	2.30	35.0	0.67	52.2
-CH_3	1.66	1.33	1.25	5.0	0.28	17.8
-OCH_3	1.67	1.39	1.20	0.17	0.13	1.3
-NO_2	3.30	2.00	1.65	0.045	0.26	0.17
Penicillin G				48.0	0.005	9600[d]
Z-Aspartame	1.32	0.11	12.0			

[a] Determined from the initial rates of hydrolysis by means of RP HPLC, isocratic elution with buffer (0.05% Et_3N,0.05% CF_3COOH,pH 2.2)/ MeOH 1:1,flow rate 2.5 ml/min and UV detection at 214 nm.

[b] Substrate concentration $0.05.10^{-3}$ - 4.10^{-3} M , enzyme concentration 3.10^{-7}M, pH7.0 (0.05 M MES buffer,0.05 M $CaCl_2$) , 25° C.

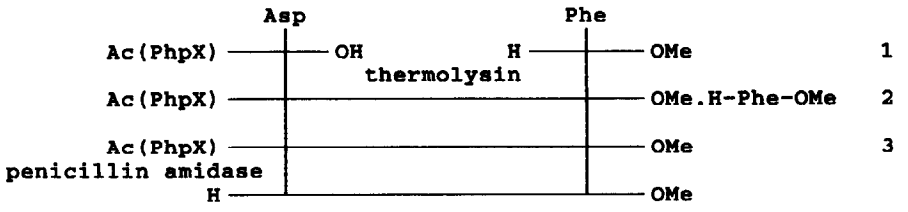
[c] Substrate concentration in the range $0.1.10^{-3}$ - 1.10^{-3} M , enzyme concentration 1.10^{-7} - 9.10^{-7}M,pH 7.8 (phosphate buffer I=0.05), 25°C

[d] Determined by A.Margolin et al. (Ref.14).

reactions involves its binding (steric effect) and the electronic nature (electronic effect)[13] . Since in the present case (Scheme 1.) the p-substituents are too far from the reaction centers,the electronic effect is negligible and the observed substituent effect can be attributed to the unfavourable S_3- P_3 steric interactions in the ground state of the thermolysin reaction (the K_m-values increase) and to the unfavourable S_1-P_1 steric interactions in the transition state of the penicillin amidase reaction (the k_{cat}- values decrease). Thus, although their reactivity is lowered, Ac(PhpX) - aspartames are still crossreactive substrates of thermolysin and penicillin amidase and can be used for a synthesis design

of aspartame with the two enzymes.

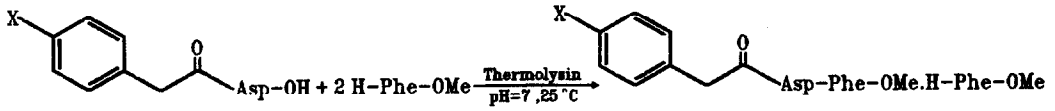
An aspartame synthesis design based on the above structure-reactivity data is shown on Scheme 2.As follows from the principle of the microscopic reversibility, the factors that control the enzyme specificity in the



SCHEME 2.

forward reaction (hydrolysis) should be valid for the reverse reaction (synthesis) as well. This means that Ac(PhpX)-Asp-OH could be coupled with H-Phe-OMe by thermolysin catalyzed condensation (step 1) the driving force of the reaction, as previously found[5],being the deposition of the product Ac(PhpX)-Asp-Phe-OMe.H-Phe-OMe[5].Since our preliminary experiments indicate that this salt is not reactive in penicillin amidase hydrolysis, the isolation of the free acid is an unavoidable step 2. Then the penicillin amidase deprotection of Ac(PhpX)-aspartame (step 3) should yield the final product.

The synthesis ,however, is a final and decisive step in the peptide/ protein engineering. It provides a feed-back information for a rational synthesis design of wild or mutated peptides/proteins.Long reaction times, side products formation and low yields may descredit even enzymic peptide synthesis.



X= H, CH_3, OCH_3, NO_2

SCHEME 3.

After 2 mmoles of Ac(PhpX)-Asp-OH[15], 8 mmoles of H-Phe-OMe[16] and 0.3 mmoles of thermolysin[17] were dissolved in 10 ml 0.05 M $CaCl_2$ and pH adjusted to ca. 6.8, the precipitation of the synthetic product Ac(PhpX)-Asp-Phe-OMe.H-Phe-OMe started within an hour (scheme 3).The conversion of the acyl component Ac(PhpX)-Asp-OH into product was followed by a reverse phase HPLC[18] until the equilibrium concentration $[Ac(PhpX)-Asp-Phe-OMe]_{eq}$ was attained. This concentration was used to calculate the maximum yield Y_{max} of the synthetic product Ac(PhpX)-Asp-Phe-OMe.H-Phe-OMe. The Y_{max} values together with that of t_{max} ,the time taken to attain of the maximum yield,are summarized in Table 2.

Table 2. Basic Parameters of the Thermolysin Catalyzed Synthesis of H-Phe-OMe Salts of Aspartame Precursors R-Asp-Phe-OMe and Physical Constants of the Corresponding Free Acids [a].

Aspartame protecting group R	$[P]_s$[b] mM	t_{max} hrs	Y_{max} %	M.p. °C	^{1}H-NMR δ[c] ppm
Ac(Ph)	46.5	46	50	135	2.49[d],2.95[e],3.33[f]
Ac($PhpOCH_3$)	37.5	40	65	122	2.50[d],2.94[e],3.34[f],3.71[g]
Ac($PhpCH_3$)	21.1	34	72	149	2.50[d],2.94[e],3.37[f],2.25[h]
Ac($PhpNO_2$)	5.4	20	83	182	2.50[d],2.96[e],3.36[f]
Z	5.6	3	88	124	2.59[d],3.00[e],5.04[i]

a) Recrystallized from methanol, elemental analysis data are in accord with the assigned structures.
b) Determined by HPLC analysis of the saturated solutions of R-Asp-Phe-OMe.H-Phe-OMe in the buffer , used thermolysin - catalyzed condensation.
c) 250 MHz, 0.01 M in DMSO-d_6 ; d) 2H, Asp βH ; e) 2H, Phe βH;
f) 2H, Ac CH_2 ; g) 3H, p-OCH_3 ; h) 3H, p-CH_3 ; i) 2H, Z CH_2

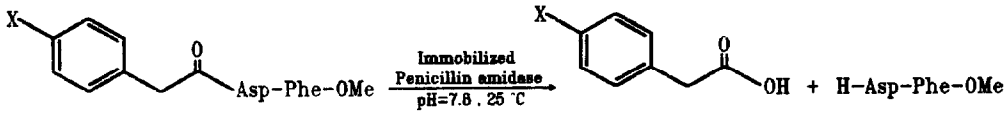
The maximum yield Y_{max} of the synthetic reaction varies with the nature of the aspartame protecting group R (Table 2). This variation does not correlate with the changes of the substrate kinetic parameters k_{cat}/K_m for the reverse reaction - the thermolysin catalyzed hydrolysis of the aspartame precursors R-Asp-Phe-OMe (Table 1 and Table 2). Moreover, no correlation is observed between the variations of k_{cat}/K_m and the maximum time t_{max}.Thus,the attainment of Y_{max} for the most reactive phenylacetyl aspartame takes the longest period of time. Therefore, the synthetic yield is controlled by a non-enzymatic equilibrium process.

The variation of the maximum yield Y_{max} ,however, correlates with the changes of the product saturation concentration $[P]_s$ (Table 2).Since

$$K_s = [P]_s$$

where K_s is the solubility equilibrium constant[19], the non - enzymic equilibrium process that controls the synthetic reaction (Scheme 3) is the precipitation of the product.

Ac(PhpX)-Asp-Phe-OMe.H-Phe-OMe was dissolved in acidic ethyl acetate the solution obtained was washed with water and dried over $MgSO_4$. After evaporation of the solvent, the resulting free acid was subjected to penicillin amidase catalyzed deprotection[20]:



X= H, CH_3, OCH_3, NO_2

Scheme 4

The conversion of the aspartame precursor into aspartame was followed by reverse phase HPLC [18].As in the case of the synthetic reaction, Y_{max} and t_{max} values for every p-substituted phenylacetyl aspartame precursors are summarized in Table 3.

Table 3.Basic Parameters of the Deprotection of p-Substituted Phenylacetyl Aspartame Precursors R-Asp-Phe-OMe , Catalyzed by Immobilized Penicillin Amidase and Physical Constants of the Resulted Aspartame.

Aspartame protecting group R	t_{max} hrs	Y_{max} %	$[\alpha]_d^{25}$[a] degrees	^{1}H-NMR δ[b] ppm
Ac(Ph)	2.5	99	+32	2.45[c],3.00[d],3.63[e]
Ac($PhpCH_3$)	3.8	90	+32	2.45[c],3.00[d],3.63[e]
Ac($PhpOCH_3$)	7.0	92	+34	2.45[c],3.00[d],3.63[e]
Ac($PhpNO_2$)	8.3	30		

a) (c 1, 1N AcOH),Lit.[21] +32; b) 250 MHz, 0.01 M in DMSO-d_6

c) 2H, Asp βH; d) 2H, Phe βH; e) 3H, Phe OCH_3

The deprotection reaction (Scheme 4) is effective only when penicillin amidase is immobilized. Free enzyme is not active probably because of substrate inhibition by p-substituted phenylacetic acid in concentrated substrate solutions[22]. The correlation of the maximum time t_{max} with the specificity constants k_{cat}/K_m (Table 1 and Table 3) suggests that, unlike the synthetic reaction, the deprotection is controlled by enzyme-catalyzed step. The maximum yield Y_{max} varies slightly with the nature of the p-substituent of the aspartame precursor, except of the p-nitro derivative. The low k_{cat}/K_m value for this precursor implies that the maximum yield is not reached since longer reaction time affords side product formation.

ACKNOWLEDGEMENTS:We are grateful to Prof. V.Kasche (Technical University Hamburg-Harburg), Dr.K.Voivodov and Dr.N.Stambolieva for discussion of the data.

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17. Purchased from Boehringer Mannheim and used without further purification.

18. Isocratic elution with buffer (0.05 % TEA, 0.05 % TFA , pH 2.2)/ MeOH 1:1 flow rate 2.5 ml/min and UV detection at 214 nm.

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20. To 5 ml 0.2 M substrate solution, 150 mg of immobilized in polyacrylamide gel penicillin amidase from Antibiotic-Razgrad (0.1 mmoles/g support) were added. The reaction mixture was shaken at 25°C and pH 7.8, was maintained with 1N KOH. The product isolation is similar to the procedure described by Fuganti, C.; Grasselli, P.; Casati, P. *Tetrahedron Lett.* 1986, *27*, 3191-3194.

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