Enzymatic esterification and transesterification of N-protected Glu or Asp and synthesis of sweetener aspartam analogues by a new glutamic acid specific endopeptidase

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Esterification and transesterification reactions are carried out in water miscible organic solvents by using a new glutamic acid specific endopeptidase, isolated from Bacillus licheniformis. Sweetener aspartam analogues syntheses are also described.

Keywords: glutamic acid specific endopeptidase (BL-GSE); esterification; transesterification; peptide synthesis; sweetener aspartam analogues

1. Introduction

Protease-mediated peptide bond synthesis, first reported by Bergmann and Fraenkel-Conrat [1], has received increasing interest due to the inherent advantages it possesses over chemically based peptide bond syntheses (for reviews, see Fruton [2] and Chaiken et al. [3]).

A Glu/Asp specific endopeptidase, very recently isolated from Bacillus licheniformis [4], belonging to the serine group and called BL-GSE, is able to catalyse the coupling of a number of different nucleophiles (H-Xxx-NH_2) corresponding to the P1′ position (Schechter and Berger notation) on Glu or Asp residues under kinetic control [5].

The effect of water miscible organic solvents has been studied in an attempt to diminish esterase activity and secondary reverse hydrolysis of the new peptide bond (dimethylformamide, methanol or acetonitrile).

2. Esterification and transesterification reactions

Transesterification reactions have been described for numerous proteolytic enzymes [6]. It has been also shown that BL-GSE catalyses transesterification when

the coupling between Z-Glu-OBzl and H-Val-NH_2 is performed in 60% MeOH/Bicine buffer: the side-formation of Z-Glu-OMe intermediate is observed.

Up to 60% MeOH, BL-GSE remains active and this medium has been used in a kinetically controlled esterification of N-blocked dipeptide Z-Ala-Glu-OH; within 15 min at pH 8.5, fraction of esterification reaches 0.7. After this time the product Z-Ala-Glu-OMe is back-hydrolysed (table 1).

The syntheses of α monoesters of Glu or Asp are chemically not so easy to achieve. Furthermore, it is well known that amino esters of trifluoroethanol and benzyl alcohol are very good starting materials for enzymatic peptide synthesis [5,7].

Thus, it seems very tempting to perform peptide couplings in a one-pot reaction starting from free carboxylic amino acid residues in two successive BL-GSE catalysed steps. The intermediate ester can be further subjected to BL-GSE catalytic aminolysis. With this new protocol in hands, it is no more necessary to use α amino ester as it is generally the case in kinetically controlled peptide synthesis. This will be of great advantage in the coupling of deprotected fragments obtained by solid phase synthesis after cleavage from the resin.

Table 1

(a) Enzymatic peptide synthesis via intermediate esterification with H-Val-NH_2 as nucleophile

Acyl donor (c = 10 mM)	Intermediate esterification yield (%)	Peptide yield (%)	Reaction time	Experimental conditions
Z-Ala-Glu-OH	0	70	2 d	60% MeOH
Z-Glu-OH	0	0	3 d	70% CH_3CN + 10% $OHCH_2$-Ph
Z-Glu-OH	10	20	1 h	60% TFE
	4	50	4 d	20% TFE + 50% CH_3CN
Z-Glu-OH	8	70	5 d	60% MeOH
Z-Glu-OH	25	30	4 d	60% MeOH + 20% CH_3CN

(b) Enzymatic peptide synthesis under kinetic control conditions with H-Val-NH_2 as nucleophile [a]

Acyl donor (c = 10 mM)	Intermediate esterification yield (%)	Peptide yield (%)	Ester hydrolysis yield (%)	Reaction time	Experimental conditions
Z-Glu-OBzl	–	52	48	1 d	70% CH_3CN +10% $OHCH_2$-Ph
Z-Glu-OMe	12 [b]	5	15	1 h 30	60% TFE
Z-Glu-OMe	–	59	41	1 d	60% MeOH
Z-Glu-OMe [c]	0	–	100	2 h	70% CH_3CN +10% $OHCH_2$-Ph

[a] [BL-GSE] = 35 nM; (H-Val-NH_2) = 1 M. The reactions are stopped when the equilibrium is reached.

[b] This intermediate arises from transesterification reaction catalysed by BL-GSE.

[c] This experiment has been performed without any nucleophile in order to show that benzylic alcohol is unable to transesterify Z-Glu-OMe.

We report in table 1a some assays in various experimental conditions using MeOH, CF_3CH_2OH (TFE) or $PhCH_2OH$ which act both as esterification reagent and organic solvent in the rate of 60–80% with H-Val-NH_2 as nucleophile.

With trifluorethanol in 40–60% the coupling does occur, but after 1 h of incubation under these experimental conditions, BL-GSE is inactivated; it is necessary to add fresh enzyme periodically in order to increase the final coupling yield. When the alcoholysis is carried out in a mixture of 20% TFE and 50% acetonitrile the yield reaches 49% in 4 days and thus, the inhibitor effect of TFE is avoided.

The best results are obtained with methanol (60% in Bicine buffer): the coupling yield reaches 70% in 5 days without enzyme inactivation. Back hydrolysis of the peptide bond is avoided because of the presence of organic solvent in large amount (60–80%).

For direct ester aminolysis with the same nucleophile (table 1b), benzylic alcohol is used in 10% amounts in a mixture of acetonitrile and Bicine buffer/$CaCl_2$ to get complete miscibility; Z-Glu-OBzl is effectively one of the best acyl donors and benzylic alcohol the best leaving group but the results prove that it is the worst esterification reagent. However, this alcohol does not inactivate the enzyme: during the coupling between Z-Glu-OMe and H-Val-NH_2 in 10% benzylic alcohol, both coupling and hydrolysis of the starting ester are observed.

3. Synthesis of aspartam analogues

The well known aspartam (H-LAsp-LPhe-OMe) is one of the good examples of industrial enzymatic peptide preparation [8]. The coupling between Z-Asp-OH and H-Phe-OMe is performed using a metalloprotease (thermolysin) in water glycerol mixtures under thermodynamic control conditions.

H-Asp-Phe-OMe can be also prepared using either α-chymotypsin or papain, belonging respectively to the serine and cystein endopeptidase group, but only if the lateral chain is protected by methyl or ethyl ester like in the chemical strategy [9]. Masking the lateral carboxylic group is needed for fitting the P1 requirement of these enzymes.

Recently Yoshpe-Besançon et al. have directly prepared aspartam although in low yields from L-Asp-OH and L-Phe-OMe using a new aminopeptidase from Staphylococcus chromogenes [10].

We report in table 2 the synthesis of some aspartam analogues catalysed by BL-GSE.

Like most of the endopeptidases, BL-GSE has preferences in P1′ position for nucleophile amides, because amide closely mimics peptide bond structure: as it is shown in table 2 and in ref. [5], H-Met-NH_2 and H-Phe-NH_2 are the best nucleophiles, but unfortunately H-Asp-Phe-NH_2, H-Asp-Met-NH_2 or their Glu analogues are not tasty.

H-Asp-Phe-OMe is a poor substrate for BL-GSE: aspartam hydrolysis (10 mM) is carried out in water Bicine buffer with a very high amount of enzyme

Table 2
Enzymatic peptide synthesis of sweetener analogs of aspartam catalysed by BL-GSE under kinetic control

Acyl donor (c = 10 mM)	Nucleophiles (c = 1 M)	Ester aminolysis yield (%)	Reaction time (min)	Experimental conditions	Sweet taste [c]
Z-Glu-OBzl	H-Phe-OMe	0	30	20% CH_3CN	–
a	H-Phe-OMe	2	90	80% CH_3CN	–
	H-Phe-NH_2	50	45	85% CH_3CN	–
	H-Phe-NH-$(CH_2)_2$-OH	25	45	70% CH_3CN	–
	H-Met-OMe	15	90	80% CH_3CN	–
	H-Met-NH_2	65	30	Bicine buffer pH 8.5	–
Z-Asp-OBzl	H-Phe-OMe	5	50	20% CH_3CN	+++
b	H-Phe-NH_2	54	75	70% CH_3CN	–
	H-Phe-NH-$(CH_2)_2$-OH	44	30	70% CH_3CN	++
	H-Met-OMe	50	90	70% CH_3CN	+++

[a] [BL-GSE] = 35 nM; [b] [BL-GSE] = 6.25 μM. The reactions are stopped when the starting acyl donor has been consumed.
[c] Relative to the N-deprotected dipeptide.

(1000 fold more than in a standard reaction) and only 5% hydrolysis is obtained so far. As H-Phe-OMe is a poor nucleophile and is gradually transformed into the dicetopiperazine derivative c(Phe-Phe), we decided to prepare other dipeptides with either more stable (H-Phe-NH-$(CH_2)_2$-OH) or active (H-Met-OMe) entering nucleophiles. Hopefully, the resulting dipeptides H-Asp-Phe-NH-$(CH_2)_2$-OH) and H-Asp-Met-OMe are sweet [11].

To avoid starting ester hydrolysis and secondary hydrolysis of the new peptide bond, synthesis are carried out in water miscible organic solvents. The best conditions (70 or 80% of acetonitrile) are depending on the solubility of the nucleophile.

BL-GSE activity is higher for Glu-X substrates than for Asp-X substrates so a lower amount of enzyme is needed for the Glu coupling. However, the acyl-enzyme intermediate with Glu is probably less "stable" than that formed with Asp and is hydrolysed faster.

In all cases, a very high excess of nucleophile is needed even if the coupling is carried out in a high amount of organic solvent to reach the equilibrium state. However, when the reaction is stopped, this excess can be easily recovered from the mixture.

After enzymatic coupling aspartam analogue can be obtained by catalytic hydrogenation on Pd/C in MeOH in a quantitative yield.

In complement to this classical enzymatic synthesis, we have applied our new protocol starting from free α-carboxylic derivatives as outlined in the precedent chapter. So Z-Asp-OH or Z-Glu-OH have been coupled to H-Met-OMe in an aqueous organic medium containing 60% MeOH and Bicine buffer. The resulting yields (respectively 60 or 25%) are even better than the best reported in table 2, obtained under classical kinetic control conditions.

Table 3
HPLC retention time [a]

Products	Retention time (min)	Products	Retention time (min)
Z-Glu-OH	6	Z-Glu-Phe-OMe	12
Z-Glu-OMe	11	Z-Glu-Phe-NH_2	10.5
Z-Glu-OBzl	16	Z-Glu-Phe-NH-$(CH_2)_2$-OH [b]	18
Z-Glu-O-CH_2-CF_3	10	Z-Glu-Met-OMe	17
Z-Glu-Val-NH_2	8.5	Z-Glu-Met-NH_2	9
Z-Ala-Glu-OH	7	Z-Asp-Phe-OMe	13
Z-Ala-Glu-OMe	15	Z-Asp-Phe-NH_2	12.5
Z-Ala-Glu-Val-NH_2	13	Z-Asp-Phe-NH-$(CH_2)_2$-OH [b]	18
Z-Asp-OBzl	16	Z-Asp-Met-OMe	18

[a] HPLC runnings are carried out on Spherisorb C18 reverse phase column with linear gradient 25–75% B over 20 mm (B = CH_3CN + TFA 0.1%; A = H_2O + TFA 0.1%), monitored at 254 nm.
[b] These new compounds have been well characterized by ^1H NMR and FAB-mass analyses.

The catalytic power of BL-GSE in peptide coupling and esterification allows the easy preparation of new aspartam analogues and of different α-amino esters in fair yield.

4. Material and methods

Material. Glu/Asp Specific Endopeptidase (BL-GSE) is isolated from Bacillus licheniformis as previously described in Carlsberg Laboratory, Denmark [4]. Z-Phe-OH, Z-Asp-OBzl, Z-Glu-OBzl, H-Phe-OMe and H-Met-OMe are from Bachem, Switzerland. All other reagents are of analytical grade. Bicine is obtained from Sigma, USA. HPLC runnings use equipment from Waters Associates, USA.

Methods. The product H-Phe-NH$(CH_2)_2$-OH is prepared as described in ref. [11]. The enzymatic reactions are carried out as follows: to the mixture of 10 mM Z-Asp-OBzl and 1 M HBr, H-Phe-NH$(CH_2)_2$-OH in 70% CH_3CN and 50 mM Bicine/2 mM $CaCl_2$ buffer is added 2.5 µl GSE solution (c = 1.2 mg/ml) at the end after having checked the pH (8–9). This mixture is stirred at room temperature. Along the reaction course, a volume of 10 µl is removed from the reaction mixture and analyzed by HPLC running.

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