Enzymatic synthesis of a CCK-4 tripeptide fragment

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Abstract: Objective Tosynthesizeatripeptidederivative Phac-Met-Asp(OMe)-Phe-NH_2,whichisafragmentofthegastrin C-terminaltetrapeptideCCK-4,byenzymaticreaction. **Methods** Threefreeenzymes, 琢chymotrypsin,papainandthermolysin fromacyldonorPhac-Met-OCamwasinvolvedinthreesteps. Thechoiceofappropriateenzymesandsolventswasselected. **Results** Allenzymaticreactions were obtained inreasonableyields (63%-92%). FAB-MS and FD-MSverifiedthecorrect molecularmassofthepeptides. **Conclusion** Studiesonthe 琢chymotrypsincatalyzedcouplingreaction betweenPhac-Met-OCam andH-Asp(OMe)$_2$ havefocusedonthelowwatercontentmedia.Bypapaincatalyzedsaponificationof Phac-Met-Asp(OMe)$_2$, 琢methylesterof asparticacidisselectivelyhydrolyzedtoretain 茁methylester, andPhac-Met-Asp (OMe)-OHandH-Phe-NH_2 canbecoupledefficientlybythermolysin.

Key words: enzymaticsynthesis;peptidebond;gastrinCCK-4;tripeptidederivative

Enzymatic approach has provedtobeapromising alternativeto chemicalmethodsinpeptidesynthesis[咱~4暫] in regardtoitsnumerousadvantagesoverthechemical ones, suchasthepromotionofpeptidebondformation under mild conditions, minimumside-chainprotection, and elimination of racemization. The use of harmful organicreagents,inaddition,istotallyavoidedtojustify thetechnicalapplicationsofthisapproach.

It is important and difficult to choose from the reaction and separation conditionswhentotalenzymatic or enzymochemicalsyntheticstrategiesare employedto produce moderate-length tolarge peptide. This fact is particularlysignificant when the convergent strategy is adoptedbecauseofthegreatnumberofthepossibilities involvedthereinthatawaitcarefulevaluation.

Thisinvestigationisdesignedtotestthepossibility of synthesizing peptidesexclusively by enzymatic methodswithreasonableyields. As thepeptidefortesting thissynthesisapproach,thetripeptidefragmentofgastrin CCK-4 was chosen that has the same C-terminal sequence as that of gastrin and also displays the same physiologicalactivitiesasthelatter[咱暫].

Enzymatic synthesis of the tripeptide derivative Phac-Met-Asp(OMe)-Phe-NH_2 is reportedinthispaper. Thephenylacetylgroup (Phac) isused astheprotection group for the amino group, and can be cleaved at the endof the synthesis with penicillin G amidase without affecting the peptide bonds[咱袁暫]. Thus, beginning with Phac-Met-Ocam (carboxamidomethyl ester), the target tripeptidederivativewassynthesizedsuccessfullywith3 freeenzymes, 琢chymotrypsin, papain and thermolysin, and all reactions werecarried out in reasonable yields (Fig.1). Thekeystepsinthesynthesisofthetripeptide werethecouplingofPhac-Met-OCamandH-Asp(OMe)$_2$ to form Met-Asp peptide bond catalyzed by 琢chymotrypsinandthe selectiveenzymatichydrolysisof 琢ester ofPhac-Met-Asp(OMe)$_2$ withpapain.

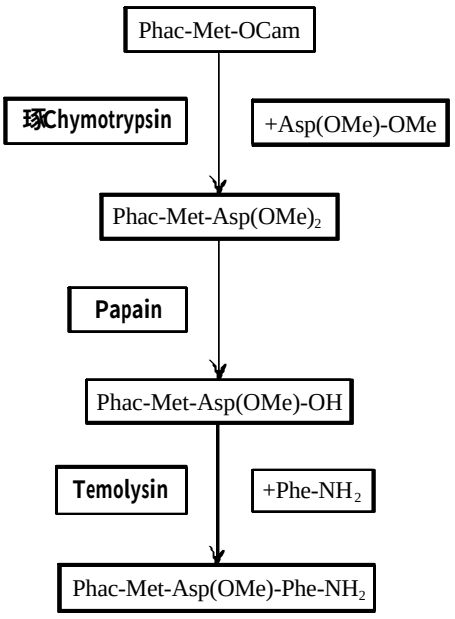

Fig.1 Enzymatic synthesis of CCK-4 tripeptide fragment

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MATERIALS AND METHODS

Materials

琢Chymotrypsin (EC3.4.21.1) frombovinepancreas (Type域 crystallized for 3 times from chymotrypsinogenthathadbeencrystallizedfor4times,dialyzed essentiallyintosalt-free and lyophilized powder, with activity40-60 U/mgof protein), papain(EC3.4.22.2) from **Carica papaya** (twice crystallized, lyophilized powder, 10-20U/mgofBAEEassay), andthermolysin (EC3.4.24.2) from **Bacillus thermoproteolyticus** rokko (crystallized and lyophilizedpowdercontainingcalcium andsodiumbuffersalts,50-100U/mgofprotein) were all from Sigma (USA). Theamino acid derivatives, Phac-Met-OCam, H-Asp(OMe)-OMeandH-Phe-NH_2窑 HCl were synthesized in our laboratory by a standard procedure. All otherreagents and solventsusedinthis studywereofanalyticalgrade.

Analysis with high-performance liquid chromatography (HPLC)

HPLC (Gilson),Column: Nucleosil100RP-18,5滋m and100mm伊 mmcolumn(Macherey-Nagel). Mobile Phase (System玉): Solvent A, 0.05 mol/L $HCOONH_4$ (pH6.5); solvent B, 80%(V/V) MeOH and20%water, elution gradient from 45% to 85% B; Mobile Phase (System II): Solvent A, H_2O (0.1%TFA); solvent B, 80% Acetonitril (0.1% TFA), elution gradient from 30% to70%B; Flowrate0.3ml/min; UV detection at 260nm

Enzymatic synthesis of the peptides

Phac-Met-Asp(OMe)$_2$ H-Asp(OMe)$_2$ (350 mg, 2.18mmol)andPhac-Met-OCam(400mg,1.23mmol) were addedintoa20-mlflask, anddissolvedwithethyl acetate (10ml) whichcontained0.05mol/LTris窑HCl buffer (pH 9.0, 150 滋). After the addition of 琢chymotrypsin (15 mg), the mixture was shaken at room temperature until the peak of Phac-Met-OCam wasnotobservedinHPLC(mobilephasesystem玉and域). Thereactionmixturewasdilutedbyethylacetate(50ml), andtheorganiclayerwaswashedwith5%Na_2CO_3, 10% citric acid and saturated NaCl solution in succession, anddriedoveranhydroussodiumsulphate. Afterconcentratedinvacuum, awhitesolidsubstancewasobtained (320mg,63%).mp128-129 益FAB-MS:411$(M+H)^+$.

Phac-Met-Asp(OMe)-OH Phac-Met-Asp(OMe)$_2$ (1.6g, 4mmol) was suspendedin0.2mol/LKH_2PO_4 buffer (pH 6.0, 50 ml) containing 2-mercaptoethanol (100 滋).ThepHofthemixturewasadjustedto6.0with 1 mol/L HCl. Papain (50 mg) was subsequently added tothesuspension, which wasshakenatroomtemperatureunderconstantpHcontrol (pH6.0) untilthepeak of Phac-Met Asp $(OMe)_2$ disappeared as observed by HPLC(MobilePhaseSystem域). ThepHofthemixture wasthenadjustedto2to3with6mol/LHCl, followed byextractionwithethylacetate(2伊00ml)andwashing the extract with 10% citric acid and saturated NaCl solutioninsuccession.Theorganiclayerwasdriedover anhydrous sodium sulphate and concentrated in vacuum, and finallyawhitepowderwasobtainedafter lyophilization(1.46g, 92%), mp141~144 益, FD-MS: 396(M^+).

Phac-Met-As (OMe)-Phe-NH_2 Phac-Met-Asp(OMe)-OH(800mg,2mmol)andH-Phe-NH窑HCl(600mg,3 mmol) were suspended in distilled water(20ml). The pH of the mixture was adjusted to 7.0 with 4 mol/L NaOH. Thermolysin(10mg)wasaddedtotheresultant cloudy solution, uponwhichwhiteprecipitatewasimmediatelyproduced. The reactionmixture was keptin 40 益 of water bath overnight. After the mixture was cooled in ice bath, the precipitate was collected by filtration, washed carefully withcold 1% $NaHCO_3$ (3伊 10ml),water(2伊0ml),and5%citricacid(3伊0ml), respectively. The precipitate was again washed with water untillitturnedneutral, and a white powder was resulted(895mg,82.6%).mp: 210~213 益, FAB-MS: 543$(M+H)^+$.

RESULTS AND DISCUSSION

The synthesis was initiated at the amino terminal of the peptide, opposite to the way generally used in chemicalpeptidesynthesis.Thechemicalsynthesisalways inheritsthedangerofracemizationduringtheactivation ofthecarboxylgroupofthepeptide,aproblemnotseen inenzymaticmethods. Thisisoneofthemajoradvantagesofenzyme-catalyzedpeptidesynthesis.

Enzymatic synthesis of Phac-Met-Asp(OMe)$_2$

Usually, itisnoteasytoformthepeptidebondof Met-Asp by means of coupling of Met and Asp derivatives catalysedbyenzymes[噎]. In theinitialapproach,

the synthesis was attempted in aqueous buffer phase or in organic solvents-buffer bycouplingofPhac-Met-OR (R=H, Me orEt)andH-Asp(OMe)$_2$ underthe catalysis by3enzymes(琢chymotrypsin, papainorthermolysin), but it was not successful, andonlythehydrolysisproductsofester(Phac-Met-OR)wereobtained.

Usingsuchactivated ester as thecarbamoylmethyl (Cam) esterastheacyldonortocontrolpeptidebond formation inthereaction kinetics, the dipeptidePhac-Met-Asp (OMe)$_2$ could be obtained through 琢chymotrypsin-catalyzed couplingofPhac-Met-OCam and free H-Asp (OMe)$_2$ inayieldabove63%, andthe reaction proceeded in organic solvent ethyl acetate containing 0.05mol/mlTris窑HClbuffer(lessthan1.5%, V/V, pH 9.0). Water is essential for the enzyme to retain its bioactivityin organic solvents咱猿曾; and adequate water activity(琢) inthesyntheticreaction systemmayfacilitate theformationofthepeptide bonds. Thedipeptide Phac-Met-Asp (OMe)$_2$ could not be obtained in reasonable yields when the content ofTris窑HCl buffer (0.05mol/ml)exceeded2%inthe organicsolventethyl acetate, andwhenthebuffercontentreached10%(V/V) in theethylacetate, a stickymixturewould result. It shouldbenotedthatnoproductcouldbeobtainedwhen thereactionwascarriedoutinwaterlessethylacetate or acetonitrileeveninthepresenceofthesameenzyme.

Fig.2 describes the kinetic time course of 琢chymotrypsin-catalyzed Phac-Met-Asp(OMe)$_2$ synthesis, in which it is manifest that the hydrolysis product (Phac-Met-OH) ofPhac-Met-OCamwas producedduringreaction. Results ofthisstudyshowedthatPhac-Met-Asp (OMe)$_2$ was stable in ethyl acetate containing 0.05 mol/mlTris窑HClbuffer (1.5%, V/V, pH9.0) inthe presence of 琢chymotrypsin within 1 or 2 days without amideoresterhydrolysisreaction.

Enzymatic synthesis of Phac-Met-Asp(OMe)-Phe-NH$_2$

The synthesis of the protected tripeptide, Phac-Met-Asp (OMe)-Phe-NH$_2$, wasaccomplishedby coupling of Phac-Met-Asp (OMe)-OH and H-Phe-NH$_2$ in a yield of 82.7% with thermolysin in water. Phac-Met-Asp (OMe)-OH was obtained by papain-catalyzedsaponificationofPhac-Met-Asp(OMe)$_2$ in 0.2 mol/ml KH_2PO_4 pH 6.0 buffer, and 琢methyl ester of aspartic acid was hydrolyzed while 茁methyl ester

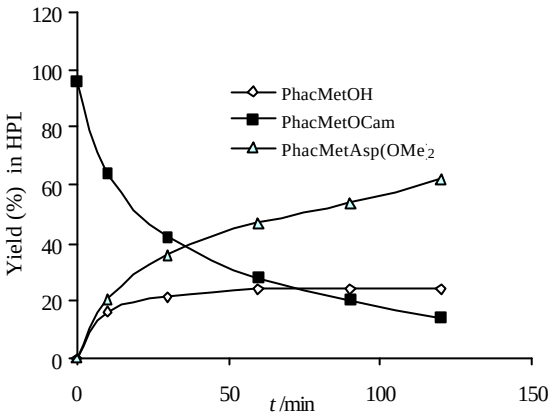

Fig.2 Time course of the enzymatic reaction between Phac-Met-OCam and H-Asp(OMe)$_2$

Thereactionwascarriedoutinethylacetatecontaining0.05mol/ml Tris-HClbuffer(1.5%, V/V,pH9.0)with 琢chymotrypsin. 姻AcyldonorPhac-Met-Ocam; 吟DipeptidePhac-Met-Asp(OMe)$_2$; 音HydrolysisproductPhac-Met-OH.

remainedsteadyunderthereactioncondition.Theresulted dipeptide, Phac-Met-Asp (OMe)-OH was important since it could easily bind H-Phe-NH$_2$ in high yield (>82.6%) inthepresenceofthermolysin, andproduced almostnoby-products. Inaddition, the tripeptidePhac-Met-Asp(OMe)-Phe-NH$_2$ wasnot obtained successfully with enzyme-catalyzed coupling of Phac-Met-Asp (OMe)$_2$ andH-Phe-NH$_2$ asshowninTab.1.

Tab.1 The results of the synthesis of Phac-Met-Asp (OMe)-Phe-NH$_2$ with enzymes catalyzed coupling of Phac-Met-Asp(OMe)$_2$ and H-Phe-NH窑HCl

Enzyme	Medium	Result
琢chymotrypsin	0.2mol/L$NaHCO_3$ buffer,pH9.5	Noproduct
Thermolysin	H_2O,pH7.0	Yield<1%
Papain	0.2mol/LKH_2PO_4 buffer,pH8.5	Yield<10%

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酶催化合成 CCK-4 三肽片段

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摘要院目的 探讨酶法合成胃泌激素 CCK-4C- 末端片段三肽衍生物 Phac-Met-Asp(OMe)-Phe-NH_2遥 方法 以 Phac-Met-OCam 为起始原料袁采用三种蛋白酶即 琢糜蛋白酶尧木瓜蛋白酶尧嗜热菌蛋白酶渊简称 chymotrypsin,papain袁thermolysin冤袁经过三步酶促反应得到目标三肽化合物曰研究了酶催化条件下 Met-Asp 肽键的形成以及天门冬氨酸双酯中 琢酯的酶催化条件下的选择性水解曰对酶以及反应介质的选择等酶促合成条件进行了研究遥结果 三步酶促反应均可得到较合适的收率渊3%~92%冤袁产物经 FAB-MS尧D-MS 质谱确认遥结论 以 琢糜蛋白酶为酶催化剂袁在含有 1.5%0.05mol/L Tris-HCl 缓冲液的乙酸乙酯介质中袁hac-Met-OCam 和 H-Asp$(OMe)_2$ 缩合可得到收率大于 63%的缩合产物曰木瓜蛋白酶催化下可选择性水解 Phac-Met-Asp$(OMe)_2$ 中的 琢酯而保留 茁酯曰嗜热菌蛋白酶能有效地催化 Phac-Met-Asp(OMe)-OH 与 H-Phe-NH_2 生成肽键遥

关键词院酶促合成曰肽键曰胃泌激素 CCK-4曰三肽衍生物