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The Evidence for Pepsin-Catalyzed Transpeptidation

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Abstract

Porcine pepsin hydrolysis of hexapeptide L-S-pNF-Nle-A-OMe in the presence of dipeptide L-L generates a new peak on HPLC analysis of reaction mixtures that is not seen when enzyme is incubated with either peptide alone. The peaks can be detected spectroscopically at either 214 or 254 nm, the latter consistent with a new peptide containing the *p-nitro*-F residue. The data suggest acyl transpeptidation between E(L-S-pNF) and L-L to form L-S-pNF-L-L. Consistent with this inference are ① the ability of L-L-NH_2 and inability of Boc-L-L to undergo a similar transpeptidation reaction, and ② the data from electrospray mass spectrum. This synthesis requires that Nle-A-L-OMe be released before L-S-pNF, an order opposite to that proposed on the basis of product inhibition kinetics. Consistent with this inference are reciprocal solvent isotope effects ; normal isotope effects of 1.736 ± 0.121 on the formation of Nle-A-L-OMe and 2.281 ± 0.184 on the formation of L-S-pNF, coupled to an inverse isotope effects of 0.576 ± 0.045 on the formation of L-S-pNF-L-L. Because transpeptidation precedes faster in D_2O, the isotopically-sensitive step must occur after release of Nle-A-L-OMe. Isotopically-enhanced transpeptidation is consistent with the Uni-Bi iso mechanism postulated on the basis of an isotope effects on Vmax but not on Vmax/Km[1] and confirmed by isotope effects on the onset of inhibition by pepstatin[2].

Key words : Transpeptidation, Pepsin

Introduction

Porcine pepsin is known to catalyze transpeptidation as well as hydrolysis, in which a C-terminal amino acyl group is transfered to a second substrate[3-5]. Although covalent intermediates have been proposed from that pepsin catalyzes both amino and acyl transpeptidation reactions, no direct evidence has not been reported[6-7]. James and Sielecki[8] showed that the formation of covalent intermediates is not likely to happen, based on the x-ray crystallography data. However, the use of relatively long incubation times raised the possibility that the analyzed products may not be the initial products but rather secondary reaction products[8]. Antonov *et al.*[9] reported that isotopic oxygen (O^{18}) from water was incorporated into the peptide bond of transpeptidation products, which supports the noncovalent mechanism. Rebholz and Northrop[1] proposed that pepsin obeys an iso mechanism in which the enzme finishes proteolysis in a different form than when it started and that the isotope effect with the substrate arises from an isotopically-sensitive step within the isomerization of free enzyme. Recently, Cho *et al.*[2] confirmed the proposed iso mechanism by using solvent isotope effects on the inhibition of pepsin by pepstatin. In this study, we tried to induce transpeptidation by manipulation of the reaction

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conditions with cognizance of the kinetics of iso mechanism in order to resolve long-standing controvercies about the chemical mechanism of aspartic proteases.

Materials and Methods

Materials

Leu-Ser-*p-nitro*-Phe-Nle-Ala-Leu-OMe, Leu-Leu-OH, Leu-Leu-NH_2 and Boc-Leu-Leu were purchased from Bachem, and D_2O from Cambridge Isotope Lab. Porcine pepsin was obtained from Sigma and μ-Bondapack HPLC column (3.9×300mm, particle size 10μm, pore size 125A) from Millipore.

Methods

Porcine pepsin was purified by a Bio-Scale Q_2 column (7×52mm) (FPLC, Bio-Rad BioLogic System) equilibrated with 0.1M acetate buffer (pH 4.0). A 0-100% NaCl gradient was used and the flow rate was 2ml/min. The purified enzyme was found to be homogenous by electrophoresis (data not shown). Separation of transpeptidation products was carried out by using a Bio-Rad HPLC 5000T system. Reaction mixture contained 1.15 nM pepsin, 1.2 mM of Leu-Ser-*p-nitro*-Phe-Nle-Ala-Leu-OMe and 6.43 mM of an alternate product inhibitor. Aliquots were taken out at time intervals and assayed at 214 nm and 254 nm for transpeptidation. A 0-70% acetonitrile gradient was used and the flow rate was 5 ml/min. Electrospray mass spectra were collected in the positive-ion mode using a VG Platform II mass spectrometer (VG Analytical, Manchester, UK) and a methanol mobile phase. The samples were dissolved in 98% formic acid and diluted with mobile phase to give a solution of 0.1 mM concentration. The solution was pumped into the mass spectrometer source (60℃) at a flow rate of 0.01 ml/min.

Results and Discussion

Transpeptidation Reactions

Transpeptidation reaction is extremely sensitive to pH and other reactant conditions. At pH 3.5, for example, where peptide bond cleavage of slow substrates is optimal and rate-limiting[10], and therefore optimal transpeptidation might be expected when the isomerization of free enzyme is made rate-limiting by a fast substrate, transpeptidation barely could be detected. At pH 4, however, one HPLC peak attributed to the transpeptidation was reproducibly confirmed and quantitatively defined, with a rate of formation 0.3% that of turnover. Incubating 1.15 nM porcine pepsin with 1.2 mM of substrate Leu-Ser-*p*-nitro-Phe-Nleu-Ala-Leu-OMe, together with 6.43 mM of Leu-Leu (P') as an alternate product inhibitor, a new peak was detected at 214 nm on HPLC, clearly distinct from Leu-Leu and products NLeu-Ala-Leu-OMe (P) and Leu-Ser-*p-nitro*-Phe (Q), as shown in Fig. 1. This peak was not present during incubations in the absence of substrate or alternate product. Moreover, the peak can also be detected at 254 nm where Nleu-Ala-Leu-OMe is nearly transparent, as shown in the bottom of Fig. 1. Hence, the new peak peptide must contain the *p-nitro*-Phe chromophore. The product of the transpeptidation reaction is therefore infered to be Leu-Ser-*p-nitro*-Phe-Leu-Leu (Q-P'). Consistent with this inference is the ability of Leu-Leu-NH_2 and inability of Boc-Leu-Leu to undergo a similar transpeptidation reaction. Fig. 2 shows that molecular weight of the new peak is 636.1. Therefore, the data from electrospray spectrum strongly supports that the new peptide is Leu-Ser-*p-nitro*-Phe-Leu-Leu (MW 636). The inference suggests the order of product release as Nle-Ala-Leu-OMe followed by Leu-Ser-*p-nitro*-Phe—exactly the opposite of the order proposed from product inhibition kinetics over two decades ago[11-12].

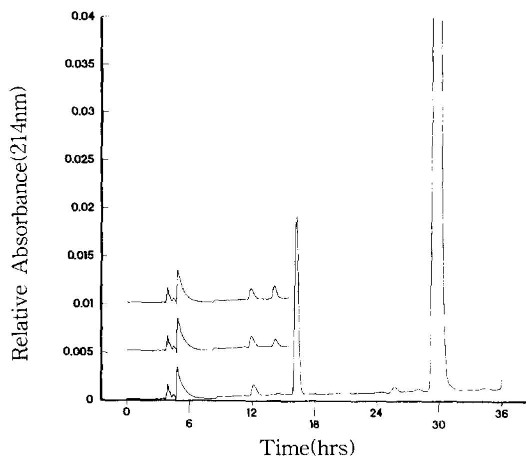


Fig. 1. HPLC elution profiles of transpeptidation incubations. The peaks and [times, min] are : solvent front [5], Leu-Leu(P') [12], Leu-Ser-*p-nitro*-Phe-Leu-Leu(Q-P') [14], Leu-Ser-*p-nitro*-Phe (Q) [16], impurities [25-27], and Leu-Ser-p-nitro-Phe-Nle-Ala-Leu-OMe [30]. Absorbancies in the top three profiles were monitored at 214 nm at 4, 8, 12 hours of incubation, respectively from the bottom.

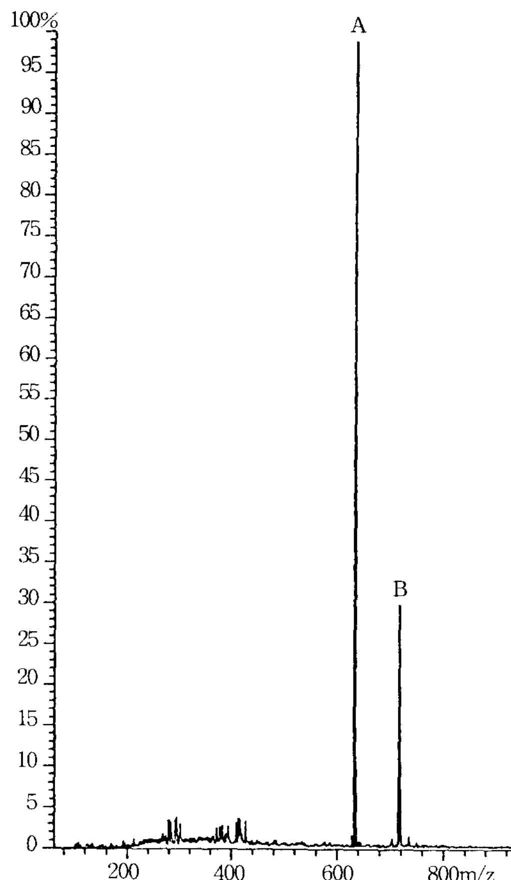


Fig. 2. Electrospray mass spectrum of the new peak from HPLC analysis of transpeptidation reaction mixture. Peak A (MW 636.1) represents the new peak from HPLC profile. Peak B (MW 716.3) represents reference compound (erythromycin).

Solvent Kinetic Isotope Effects

Initial velocities of peptide hydrolysis and transpeptidation were measured at pH 4 in H_2O and compared to similar results at pD 4 in D_2O. The results are shown in Table 1. The solvent kinetic isotope effect on the formation of Nleu-Ala-Leu-OMe by peptide hydrolysis is 1.736 ± 0.121 and represents the isotope effect on catalytic turnovers in the presence of inhibitory Leu-Leu-OH. In contrast, the solvent kinetic isotope effect on the formation of Leu-Ser-*p-nitro*-Phe-Leu-Leu by transpeptidation is 0.576 ± 0.045. The inverse effect on transpeptidation means that transpeptidation proceeds faster in D_2O, consistent with the isotopically-sensitive step occuring downstream from the Leu-Leu binding step. How much faster? The reciprocal of 0.576 is 1.736 — *precisely the magnitude of the kinetic isotope effect on catalytic turnover*! The precision of agreement supports four important conclusions :

① **Turnover and transpeptidation are catalyzed by the same enzyme** : Previous reports of transpeptidation with pepsin[13] and penicillopepsin[14] relied on either NMR or radioactive tracers to trace levels of products, which allows for the worrisome possibility of synthesis by a minor impurity of a different enzymes.

Table 1. Pepsin-Catalyzed Transpeptidation Kinetics

Product	V_H*	V_D*	^DV
L-S-pNF	19.532 ± 0.963	8.563 ± 0.482	2.281 ± 0.181
Nle-A-L-OMe	18.910 ± 0.811	10.891 ± 0.472	1.736 ± 0.121
L-S-pNF-L-L	0.430 ± 0.022	0.747 ± 0.032	0.576 ± 0.045

*Relative change in absorbance

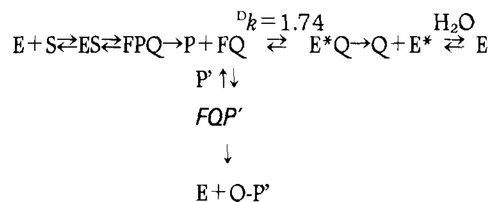
② **Reprotonation may be kinetically significant with slow as well as fast substrates** : Had the reprotonation step come after the relase of both products, then it would have been independent of the composition of the substrate and therefore kinetically insignificant with slow substrates. Instead, reprotonation is coupled to product release and therefore sensitive to peptide composition.

③ **The isotopically-sensitive step further downstream than relase of the second product, Leu-Ser-*p*-*nitro*-Phe** : If second product release preceded the isotopically sensitive step there would be no basis for a solvent isotope effect on transpeptidation.

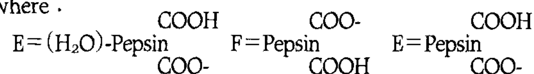
④ **The order of steps downstream from hydrolysis of the sissle peptide bond has little or no randomness** : To the extent that some of the second product might escape before the isotopically-sensitive step, isotope effect on transpeptidation would become closer to one and its reciprocal would no longer precisely match the isotope effect on catalytic turnovers.

Kinetic Mechanism

These data and inferences expand the kinetic mechanisim for pepstatin inhibition[2], in which product release steps had been combined for simplicity. Solvent kinetic isotope effects on pepstatin inhibition and transpeptidation dictate the following kinetic mechanisim for transpeptidation and turnover :



where :



The solvent isotope effects and inferences also eliminate an alternative hypothesis for the origin of the solvent isotope effect : protonation of the sissle amino group to facilitate the dissociation of the second product. The transpeptidation results show that the sissle amino group leaves with the first product, not the second ; if its slow protonation were to give rise to a solvent isotope effect, that effect would then have to be expressed on both V and V/K, whereas only ^DV is significant[1]. The hypothesis that reprotonation of the two aspartic carboxylic groups in the active site of pepsin was responsible for the solvent isotope effects with fast substrates was originally[1] based upon an analogy with proline racemase kinetics [15] ; now it is the only hypothesis remaining that is consistent with the data.

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초록 : 펩신촉매에 의한 Transpeptide의 생성

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HPLC 및 electrospray mass spectrum으로부터 L-L dipeptide의 존재하에서 pepsin은 hexapeptide인 L-S-pNF-Nle-A-OMe를 가수분해하여 가수분해물외의 새로운 생성물을 합성하는 것이 확인되었다. 이 생성물은 254nm에서 *p-nitro*-Phe 잔기를 포함하는 peptide였다. 실험결과로부터 E(L-S-pNF)와 L-L 사이의 acyl transpeptidation에 의해 L-S-pNF-L-L가 생성됨을 추론할 수 있다. L-L-NH_2는 transpeptidation이 되나 Boc-L-L는 되지않는 사실은 상기추론을 뒷받침한다. 이러한 transpeptidation 결과는 product 저해실험에 의한 결과에 기초한 것과는 반대로 L-S-pNF가 해리되기전에 Nle-A-L-OMe가 먼저 한다는 것을 보여준다. 그리고, electrospray mass spectrum으로부터 위에서 검출된 새로운 펩티드에 해당하는 peak (MW 636.1)을 얻었는데, 이는 새 펩티드의 생성을 확실히 증명하는 증거이다. 한편, Nle-A-L-OMe 생성에 대한 solvent isotope effect는 1.736±0.121이며 L-S-pNF는 2.28±0.184 그리고 L-S-pNF-L-L의 생성에는 inverse isotope effect로서 0.576±0.045였는데, 이는 상기 생성물 해리 순서를 확인시켜 준다. D_2O에서 transpeptidation은 더 빠르기 때문에 isotopically-sensitive단계는 Nle-A-L-OMe 해리후에 존재하는 것을 알 수 있다. 본 실험결과는, Rebholz and Northrop[1] 및 Cho 등의[2] iso-mechanism이론의 타당성을 제시한다.