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N-terminal domain of pepsin as a model for retroviral dimeric aspartyl protease

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Abstract. *Autolysis of porcine pepsin at pH 4 affords a derivative possessing intrinsic proteolytic activity. This derivative was isolated by alumina pseudo-affinity chromatography and gel-filtration and was found to result from the tight association of two identical molecules, 135 amino acids long, emerging from the N-terminal domain of pepsin. This finding emphasizes a similarity with the only aspartyl-proteases known to act as dimers, the retroviral proteases.* © 1990 Academic Press, Inc.

Pepsin and in general non-viral aspartyl proteases are composed of two domains, each contributing to the formation of the active site. These enzymes are thought to have evolved from an ancestral protein by gene duplication and subsequent gene fusion (1). They are characterized by two triad of amino acids, *Asp-Thr-Gly* , highly conserved and located in each topologically similar lobe. This suggested that, prior to gene duplication, the primordial enzymes may have acted as dimers. The recently presented evidence that retrovirus encode half an aspartyl protease (2,3), active as a dimer (4,5,6), strengthened this hypothesis.

Studies to determine to what extent the entire polypeptide chain of the aspartyl-proteases is needed to achieve activity were reported on pepsin and on renin. The experiments to shorten the pepsin molecule were achieved by autodigestion (7,8) in an attempt to isolate from the enzyme the smallest possible fragment retaining activity, the "active site". Smaller renin fragments, still active, were isolated from juxta-glomerular cell tumor and became apparent only under strong dissociating conditions. Their presence was attributed to limited proteolytic attack on the peptide connecting the two domains of the protein (9).

This study was undertaken to learn if limited proteolysis of pepsin might result in the formation of a single domain acting as the viral aspartyl protease. A 135 amino acid fragment from the N-terminal domain of porcine pepsin was obtained by controlled autolytic degradation. It has a strong tendency to self-assembly, resulting in an active dimeric form with a specificity toward substrates and inhibitors similar to the parent enzyme.

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Materials and Methods

Porcine pepsin was obtained from Boehringer (Mannheim, Germany) as a lyophilized product and was used without prior purification. Haemoglobin (Hb), Leu-Ser-Phe(NO2)-Nle-Ala-Leu-OMe, Carboxypeptidase A and Leu-aminopeptidase were from Sigma Chemical Co. (St. Louis, MO). CPC alumina carrier (30-45 mesh) was from Fluka (Buchs, Switzerland) and all other chemical compounds were of the best commercially available grade.

Conditions of proteolysis. 25 ml of porcine pepsin solution ($3 \cdot 10^{-4}$ M) was allowed to autodigest inside a dialysis bag (Visking tube, Union Carbide, ϕ = 33 mm) immersed in acetate buffer 0.1M, pH 4, two volumes of the outer solution per volume of the inner one. The dialysis bag (200 mm long) is mantained by a glass tube (ϕ = 13 mm) and immersed in an outer cylindric container (ϕ = 20 mm): the apparatus is kept under mild agitation. The incubation was carried out at two temperatures (4°C and 25°C) subsequently. The permeable autolysate can gradually move into the outer solution, where at intervals aliquots were removed and assayed for proteolytic activity on Hb and on the synthetic substrate Leu-Ser-Phe(NO2)-Nle-Ala-Leu-OMe, as described elsewere (10,11). Samples were also taken from the dialysate for measures of protein concentration. At the end point of the autolysis (48 hrs) the dialysate, which represented 15-20 % of the starting protein concentration inside the dialysis bag, was lyophilized.

Isolation of autolytic fragment. The lyophilized dialysate was dissolved in 1 ml of acetate buffer pH 4.2, 4°C and allowed to react with 0.3 to 0.5 ml of CPC alumina (wet powder) until the loss of activity of the supernatant is nearly complete. The active material is then eluted with 0.1 M phosphoric acid (pH 4) as already described (12,13), reduced in volume and chromatographied on Sephadex G 75 and on Superose 12 in 0.05 M acetate buffer pH 4.5, standardized with various proteins of known MW, as recommended by the supplier (Pharmacia, Uppsala, Sweden). The fractions containing a protein peak coincident with the activity were pooled and lyophilized. The N-terminal and the C-terminal determinations were performed by chemical and enzymatic methods. The dialysate was hydrolyzed 22, 24, 30 hrs in HCl and analysed on a Picotag System (Waters-Millipore, Milford, USA), according to the manufacturer's instructions.

Results and Discussion

Porcine pepsin ($3 \cdot 10^{-4}$ M), dialysed against twice the volume of buffer (pH 4) under controlled conditions of temperature and pH (Fig.1), undergoes spontaneous degradation and dialysable autolytic fragments, which show both an absorption at 280 nm and proteolytic activity, are released. In order to obtain a reasonable yield of active material a number of parameters such as the pH, the temperature, the concentration, the volume of the sample and the time, required optimisation.

The pH value was determined experimentally to result in an autolytic reaction sufficiently slow to avoid explosive degradation and sufficiently rapid to achieve reasonable yields. The autolysis was started at 4°C, a temperature which allowed a controlled cleavage only at the most exposed polypeptide segments and was then elevated to ensure a better overall yield of the active fragment. If the autolytic cleavage was performed at 4°C the yield was low (1% to 3%), if the temperature was enhanced to 25°C from the start no activity was obtained in the dialysate, possibly as a result of unspecific degradation. Times longer than 48 hrs were unsuitable since a loss in yield was always observed. The concentration of pepsin (10 mg/ml) is the best compromise since it must act as an enzyme and as a substrate and the choice of the volumes was made to insure the best possible drain of active material from the bag. Finally the glassware must be of good quality to avoid losses by adhesion. The first event to take place should be a limited proteolytic attack on the connecting peptide between the two

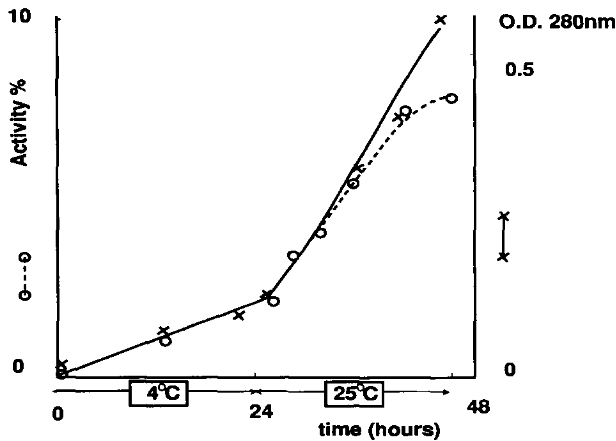

Figure 1: Autodigestion of porcine pepsin.
The absorbance and proteolytic activity of the dialysate was measured on Hb and Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe as a function of time.

domains, an assumption verified experimentally in a number of other enzymes containing substructural domains (14,15,16,17). On the basis of the dialysis bag pore size, the theoretical Mr of the molecules able to leave it should not exceed 14 000/16 000. Since the Mr of the N-terminal domain of porcine pepsin is about 16 000 and of the C-terminal one about 18 000, the resulting fragments could escape through the dialysis bag, if partly degraded.

Porcine pepsin is a phosphoprotein (18), with a phosphorylated serine at amino acid 68 in the first domain. The chromatography on alumina, pH 4.2, of the dialysed material (Fig. 2) showed that practically all the activity was retained on aluminium oxide and can be displaced by inorganic phosphate. After desalting, the product eluted from the molecular sieve chromatography as a symmetrical peak in the range of 30 000 MW and the experimental amino acid composition compared well with that calculated from the known amino acid sequence of the N-terminal domain of the parent porcine pepsin (Fig. 3). The absence of basic amino acids (present only in the C-terminal domain of porcine pepsin) ruled out the presence of the second domain, which is either more slowly or not dialysable, more susceptible to a disruptive fragmentation, or is frontally eluted during the alumina chromatography. Chemical and enzymatic analysis of the N-terminal and C-terminal allowed the identification of the active molecule as the 16-151[1] fragment from the parent pepsin (18). The actual Mr of this fragment being about 15 000 with only one *Asp-Thr-Gly* triad per molecule, the only possibility to account for the experimental MW of 30 000 together with the need of two

[1] In some of the preparations a secondary cleavage point appeared in position 9-10 of the pepsin molecule (Fig.3), yielding the principal fragment 16-151 toghether with a fragment 9-151, although in much lower quantity.

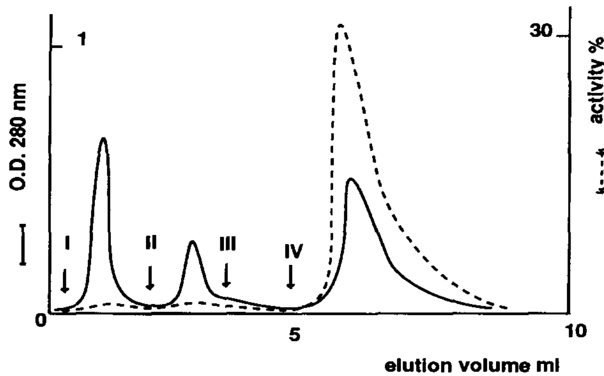

Figure 2: Alumina pseudo-affinity chromatography of the dialysate .
The column was sequentially eluted with: I, 0.1M acetate buffer, pH4.2; II, 0.1M acetate buffer, pH 4.2, 0.5M in NaCl ; III, water; IV, 0.1M phosphoric acid (pH4).

aspartates for enzymatic activity is a dimerisation of the fragment. A summary of the isolation and identification of the active fragment is presented in Table 1 .

The digestion of Hb and of the synthetic substrate Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe by the active fragment was studied and the results compared with the proteolysis by the parent enzyme. It soon appeared that Hb, which is an excellent substrate for pepsin, is a very poor one for the autolytic fragment, the kcat being lower than one order of magnitude. The synthetic peptide, in constrast, is a much better substrate (kcat/Km p.pepsin = 5 mM^{-1} s^{-1} (11) ; kcat/Km autolytic fragment = 240 mM^{-1} s^{-1}).
The low activity against the protein substrate may be taken as an indication that the dialysable fragment is unable to form the secondary bonds which are involved in the digestion of a high molecular weight substrate. The pH dependence of the enzymatic catalysis is significantly the same (optimum pH : 2.2 on hemoglobin, 4.5 on synthetic substrate). Pepstatin inhibited the digestion of the synthetic substrate with a Ki of 10^{-11} M, which further demonstrates that the dimeric fragment acts as an aspartyl protease.

One of the more striking dissimilarities between pepsin and its autolytic fragment was the better resistance of the dimer to basic pH and to autolysis. A solution of the fragment at pH 8, incubated for 120 min at 25°C, retains full activity while in the same conditions, the parent porcine pepsin significantly (70 to 90 %) inactivated. It is

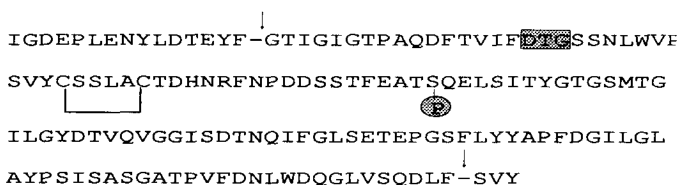

Figure 3: First domain of porcine pepsin (18)
(Shadowed the active triad and the phosphorylated Ser.)

Table 1. Purification of the active fragment from porcine pepsin

Preparation	total mg protein	Hb activity (Absorbance/min)	SA	peptide activity (Absorbance/min)	SA	Yield %
Dialysate, pH4	10	2.45	0.24	100	0.1	--
Alumina psedo-affinity chromatography	1.5	2.1	1.4	85	56	85
Molecular sieve chromatography	0.03	0.86	28	25	833	25

Isolation of the autodigestion products from 100 mg of porcine pepsine. The discrepancy between the specific activities (SA) measured on Hb and on the synthetic peptide are due to the preference shown by the active fragment for small MW substrates. The N-terminal sequence of the final product was NH2Gly-Thr-Ile-, the C-terminal one was -Asp-Leu-PheOH (see Figure 3) and the amino acid analysis in excellent agreement with the composition of fragment 16-151 from porcine pepsin.

not possible at this point to correlate this pH dependent behaviour with the structure of the two molecules: a comparison of the respective 3D structures and interactive forces is needed to deepen our understanding. Once the dimer is formed, the active fragment retains its activity for days even in solution (pH 4.5, 4°C). As pointed out above, the fragment activity was far more efficient toward smaller than higher molecular weight substrates, hence the lack of self-digestion. The stability of the dimeric fragment was not affected by ionic strength (NaCl M), urea (up to 3 M) and SDS (0.5 %) : only dioxane (5,10 and 15%) lead to a decrease in activity (12, 30 and 50 % loss). It can be asked at this point why pepsin autodegrades to complete or nearly complete inactivity. Since an autolytic dimeric fragment more resistant to autolysis is formed, a steady state should appear and from that point on the autodegradation rate should be significantly lower. The answer could be that, at the optimal conditions of pepsin digestion (pH 2-3, temp.> 4°C) the yield of the fragment is so low and its half-life so short that it will not be able to accumulate and its presence would go unnoticed.

These active fragments could be seen as models of retrovirus aspartyl proteases, which have been shown to act as dimers, and could also facilitate the search of new substrates and new inhibitors, provided the necessary adjustment for substrate specificity are subsequently made, a search sometimes hindered by the difficulty in obtaining sizable quantities of pure material. Importantly, the evidence presented here shows that mono-domain aspartyl proteases not only exist in retroviruses but that they can also be obtained by controlled cleavage of a bi-domain aspartyl protease, thus arising the question of their nomenclature. In fact there is no genetically coded retrovirus protease as such, there is only half of it and there are no active fragments from bilobal aspartyl proteases. There are, in both cases, inactive molecules which gain their activity by dimerisation. These polypeptides, bearing only half of the two necessary

active triads *Asp-Thr-Gly* , have a catalytic power which is only potential until dimerisation occurs. Thus they are (and should be called) mono-aspartyl-proteases and the fragment presented in this work is a mono-aspartyl protease (porcine pepsin).

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References

1) J. Tang,M.N. James, I.N. Hsu, J.A. Jenkins & T.L. Blundell, **Nature** 271, (1978) 618-621
2) L.H. Pearl & W.R. Taylor, **Nature** 329, (1987) 351-354
3) I. Katoh, T. Yasunata, Y. Ikawa & Y. Yoshinaka, **Nature** 329, (1987) 654-656
4) M.A. Navia, P.M.D. Fitzgerald, B.M. Mc Keever, C.T. Leu, J.C. Heimback, W.K. Heber, J.S. Sigal, P.L. Darke & J.P. Springer, **Nature** 337, (1989) 615-620
5) I.T. Weber, M. Miller, M. Jaskolski, J. Leis, A.M. Skalka & A. Wlodawer, **Science** 243, (1989) 928-931
6) A.Wlodawer, M. Miller, M. Jaskolski, B.K. Sathyanarayana, E. Baldwin, I.T. Weber, L.M. Selk, L. Clawson, J. Schneider & S.B.H. Kent, **Science** 245, (1989) 616-621
7) G. E. Perlmann, **Nature** 46, (1954) 406
8) M. Funatsu & K. Tokuyatsu, **J. Biochem** 46, (1959) 1441-1451
9) F.X. Galen, C. Devaux, T. Guyenne, J. Menard & P. Corvol, **J. Biol. Chem.** 254, (1979) 4848-4855
10) B.Kassel, C.L. Wright & P.H. Ward (1976) in*Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, ed.) pp. 452-458 Acad.Press, New York, SanFrancisco, London.
11) P. Martin, M. Raymond, E. Bricas & B. Ribadeau-Dumas, **Biochem. Biophys. Acta,** 612 (1980) 410-420
12) M-A. Coletti-Previero, M. Pugnière, H. Mattras, J.C. Nicolas & A. Previero **Biosc. Rep.** 6 (1986) 477-483
13) M-A. Coletti-Previero & A. Previero, **Anal. Biochem.**180, (1989) 1-10
14) H. Neurath (1980) in ***Protein Folding*** (R. Jaenieke ed.) pp.501-524 Elsevier/North Holland Biochem. Press, Amsterdam, New York
15) C. Vita, D. Dalzoppo & A. Fontana, **Biochemistry** 24, (1985) 1798-1806
16) A. Fontana, G. Fassina, C. Vita, D. Dalzoppo, M. Zanai & M. Zambonin, **Biochemistry** 25, (1986) 1847-1851
17) H. Neurath (1987) in ***Methods in Protein Sequence Analysis*** (K.A. Walsh, ed.) pp. 3-19 Humana Press, Clifton N.J.
18) P. Sepulveda, J. Jr. Marciniszyn, D. Liu & J. Tang, **J. Biol. Chem.** 250, (1975) 5082-5088