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HYDROLYSIS OF THE SYNTHETIC CHROMOPHORIC HEXAPEPTIDE Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe CATALYZED BY BOVINE PEPSIN A

DEPENDENCE ON pH AND EFFECT OF ENZYME PHOSPHORYLATION LEVEL *

PATRICE MARTIN

Laboratoire de Recherches de Technolgie Laitière, Institut National de la Recherche Agronomique, 65, rue de Saint-Brieuc, 35042 Rennes Cedex (France)

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Steady-state kinetic parameters for the hydrolysis of the chromophoric hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe catalyzed by bovine gastricsin and pepsin A were determined. It was shown that the phosphate content of bovine pepsin A is without any significant effect on that parameters. At pH 4.7, the specificity constant (k_{cat}/K_m) was 2455 and 2150 $mM^{-1} \cdot s^{-1}$ for the most phosphorylated bovine pepsin A (2.58 phosphate groups per molecule), before and after treatment by potato acid phosphatase, respectively. The k_{cat}/K_m ratio found for bovine gastricsin (1314 $mM^{-1} \cdot s^{-1}$) was closer to that of bovine pepsin A than that previously reported for chymosin (25 $mM^{-1} \cdot s^{-1}$). The spectral properties of the chromophoric tripeptide Leu-Ser-Phe(NO_2) in the pH range 1–3.6 were investigated. We have shown that the hexapeptide hydrolysis could be followed by difference spectrophotometry at 295 nm ($\Delta\varepsilon = -235$ $M^{-1} \cdot cm^{-1}$ at pH 1.0) thus allowing to study the effect of pH on bovine pepsin A activity in a pH range which could not be explored earlier. The pH-dependence of k_{cat}/K_m ratio of unphosphorylated bovine pepsin A indicated that enzyme activity was dependent upon the ionization of two groups of the enzyme whose pK are 1.2 and 5.0. These pK values strongly suggest the involvement of two carboxyl groups probably corresponding to the two reactive aspartyl residues (Asp_{32} and Asp_{215}) identified through active site-directed reagents for all the aspartic proteinases so far tested.

Introduction

The chromophoric synthetic hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe was first proposed by Raymond et al. [1] as a reference substrate for proteolytic assay of chymosin and bovine pepsin A. A method based on its use, for determining these two major proteinases occurring in commercial extracts of bovine abomasa, was recently suggested [2]. The amino acid sequence of this peptide is related to the primary structure of bovine κ-casein on each side of the sensitive peptide bond: Phe_{105}-Met_{106} whose cleavage triggers the milk-clotting process [3]. This peptide is also a good substrate for porcine pepsin [4,5], bovine gastricsin [6] and for *Mucor miehei* and *Mucor pusillus* acid proteases [7]. All these proteinases which belong to the class of the 'aspartic proteinases' (E.C. 3.4.23), were shown to catalyze specifically the hydrolysis of the Phe(NO_2)-Nle peptide bond in the hexapeptide.

Bovine pepsin A displays microheterogeneity related to its phosphate content [8,9]. It was re-

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solved by isoelectric focusing into four [10] to five [9] active components with phosphate contents likely ranging up to 3 mol per mol. The influence of the phosphate content on the enzymic properties of bovine pepsin A has received little attention [8,11]. It was shown that the occurrence of phosphate group(s) on bovine pepsin A more or less strongly affects its activity according to the substrate and its environment, this being particularly obvious as far as κ-casein was concerned [11]. In order to get additional information on the effect of the phosphorylation level of bovine pepsin A, steady-state kinetic parameters k_{cat} and K_m for the hydrolysis of the hexapeptide were determined for bovine pepsin A with various phosphate contents. To allow comparison with the other aspartic proteases so far tested, experiments were carried out at pH 4.7.

Studies on the pH dependence of porcine pepsin catalyzed hydrolysis of synthetic substrates, such as *N*-acetyl-L-phenylalanyl-L-tyrosine methyl ester, suggested that two carboxyl groups of apparent p*K* values 1 and 4 were involved in its catalytic mechanism [12–14]. Raymond and Bricas [15] reported that bovine chymosin catalyzed hydrolysis of the hexapeptide depended on the ionization of two carboxyl groups the p*K* values of which were 3.3 and 5.7. To determine the apparent p*K* values of the ionizable groups involved in bovine pepsin A catalytic mechanism and to avoid any effect of phosphate on that values, the pH dependence of unphosphorylated bovine pepsin A-catalyzed hydrolysis of the hexapeptide was also studied. However, pH affects the absorption spectrum of the Leu-Ser-Phe(NO_2) tripeptide resulting from the hexapeptide hydrolysis [4,2]. For pH values lower than 2.0, and thus in conditions where the free carboxyl of Leu-Ser-Phe(NO_2) is protonated (p*K* = 3.5), no difference in absorbance was found between the hexa- and the tripeptide at 310 nm, the wavelength hitherto used [2]. The spectral properties of the chromophoric probe, involved or not in a peptide bond, was then investigated in the pH range 1–3.6. To complete our study dealing with bovine aspartic proteinases, bovine gastricsin, the third proteolytic component secreted by the abomasal mucosa, was considered and its steady-state kinetic parameters determined at pH 4.7 with the aim of comparing them with those obtained for chymosin and bovine pepsin A.

Materials and Methods

Enzymes

Whole bovine pepsin A and its fractions: A_0 and A_3 which theoretically contain zero and three phosphate groups per molecule, respectively, and bovine pepsins A treated by potato acid phosphatase were prepared exactly as described elsewhere [9]. Homogeneity of these preparations was checked by isoelectric focusing as previously described [11]. Whole bovine gastricsin was prepared as previously reported [6].

Concentration of the enzyme solutions in 50 mM sodium phosphate buffer (buffer 1), pH 6.0, was determined spectrophotometrically using the following absorption coefficients: $\varepsilon_{1\,cm}^{1\,mg\cdot ml^{-1}} = 1.48$ at 280 nm [8] for all bovine pepsin A preparations and $\varepsilon_{1\,cm}^{1\,mg\cdot ml^{-1}} = 1.35$ at 277.5 nm [6] for bovine gastricsin. The scattered light was taken into account.

Peptide

The chromophoric hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe was purchased from Bachem Co. (Marina Del Rey CA, USA).

Kinetic experiments

Evolution of the difference spectrum between Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe and Leu-Ser-Phe(NO_2) in the pH range 1–3.6. 40 ml of 0.2 mM peptide solution were prepared by dissolving the crystallized peptide in 0.1 M glycine, 0.1 M NaCl buffer pH 3.6 over 16 h at 4°C. After filtration on a Millipore filter (0.22 μm), the concentration of the solution was spectrophotometrically determined using the absorption coefficient $\varepsilon = 8300\ M^{-1}\cdot cm^{-1}$ at 279.5 nm [7]. The solution was divided into 2 × 19.6 ml fractions. 400 μl of 0.1 μM bovine pepsin A in buffer 1 were added to one fraction ('products') and 400 μl of buffer 1 to the other ('substrate') which was the reference. After 2 h of incubation at 30°C in the dark, each fraction was divided into six aliquots whose pH values were adjusted by adding the same volume of HCl solutions of previously determined molarity to cover the pH range 1–3.6. Thus, for each pH, a 'substrate' solution and a 'products' solution were obtained and the difference spectra (products vs. substrate) were established from 350 to 230 nm.

The pH 3.6 couple (substrate-products) was used to check that buffering species were without any effect on spectral properties by comparing with 0.1 M sodium acetate buffer, pH 3.6.

Evaluation of kinetic parameters. The kinetic parameters, k_{cat} and K_m, were determined for bovine pepsin A preparations and for bovine gastricsin, at pH 4.7 and 30°C from initial velocity measurements using Eisenthal and Cornish-Bowden plots [16] at 5–8 substrate concentrations ranging between 13 and 290 μM. Experimental conditions were as indicated elsewhere [7] except that bovine gastricsin, which is very unstable, was in 50 mM sodium phosphate/0.2% (w/v) bovine serum albumin buffer, pH 6.0.

The pH-dependence study with unphosphorylated bovine pepsin A (A_0) was performed at 11 pH values ranging from 1.0 to 5.5 using the same buffer, i.e., 0.1 M disodium citrate adjusted to the desired pH by adding 0.1 M HCl or 0.1 M NaOH. At pH below 2.5 and above this pH value, $\Delta\varepsilon^{pH}_{295\,nm}$ and $\Delta\varepsilon^{pH}_{310\,nm}$, respectively, were used to determine the amount of peptide hydrolyzed. Experimental data were analyzed according to Tipton and Dixon [17] by plotting $\log k^{pH}_{cat}/K^{pH}_m$ against pH. Assuming that catalytic activity of unphosphorylated bovine pepsin A depends on two ionizable groups, as it is established for porcine pepsin and for all the aspartic proteinases hitherto examined by pH-dependence kinetic studies [12–15] as well as by inhibitory studies carried out with active site directed reagents [18], the following scheme can be considered:

$$\begin{array}{ccc} E^{-2} + S \rightleftharpoons ES^{2-} & & \\ K_{e_1}\ 1\downarrow \qquad\qquad 1\downarrow\ K_{es_1} & & \\ EH^- + S \rightleftharpoons EHS^- & \xrightarrow{k_{cat}} & EH^- + P \\ K_{e_2}\ 1\downarrow \qquad\qquad 1\downarrow\ K_{es_2} & & \\ EH_2 + S \rightleftharpoons EH_2S & & \end{array} \qquad (1)$$

From such a scheme, the following equation can be deduced:

$$\log \frac{k^{pH}_{cat}}{K^{pH}_m} = \log \frac{k_{cat}}{K_m} - \log\left(1 + \frac{[H^+]}{K_{e_2}} + \frac{K_{e_1}}{[H^+]}\right) \qquad (2)$$

Eqn. 2 allows the evaluation of the theoretical pK_{e_1} and pK_{e_2} of the enzyme groups involved in the catalysis.

Results

Effect of pH on the difference spectrum

From pH 1.0 to pH 2.5, the only variation between the spectra of the hexa- and tripeptides was a slight hypochromic effect leading to a difference with a negative $\Delta_{absorbance}$ between 350 and 260 nm, reaching its maximum between 295 and 280 nm according to the pH. Above pH 2.5 a bathochromic shift occurred for the tripeptide spectrum, leading, in the difference spectrum, to a positive $\Delta_{absorbance}$ whose maximum was around 307–310 nm and which increased with pH (Fig. 1).

To investigate bovine pepsin A-catalyzed hydrolysis of the hexapeptide below pH 2.5, taking into account the spectral study, we have finally chosen to follow the cleavage of Phe(NO_2)-Nle bond by recording $\Delta_{absorbance}$ at 295 nm and by using $\Delta\varepsilon^{pH}_{295\,nm}$ whose variation as a function of pH is shown in Fig. 2.

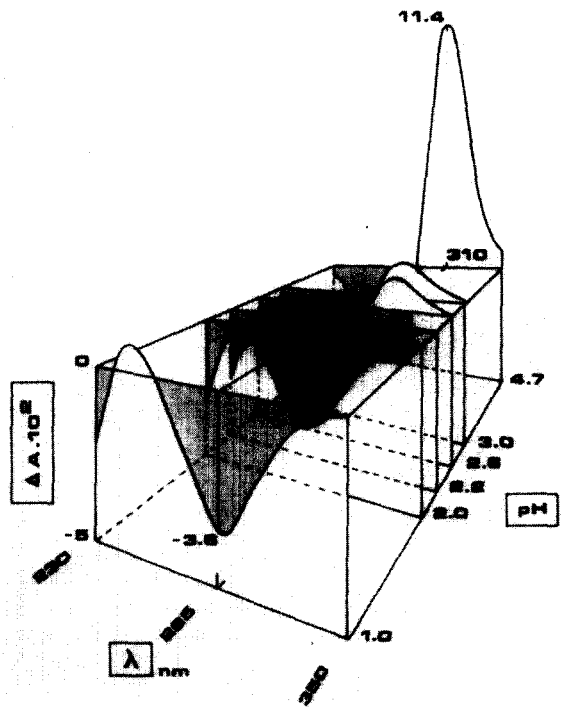

Fig. 1. Effect of pH on the ultraviolet-difference spectrum between Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe and Leu-Ser-Phe(NO_2).

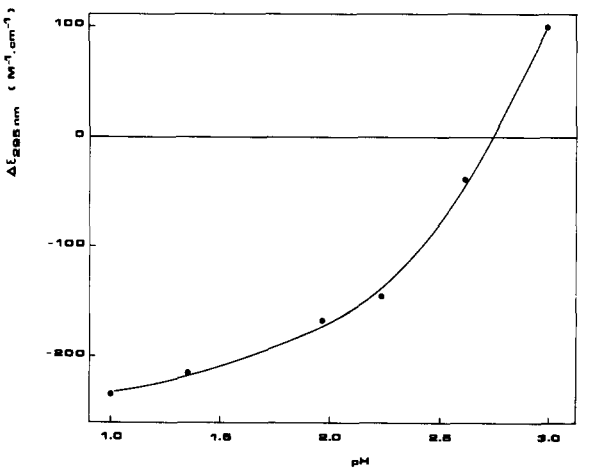

Fig. 2. Variation with pH of the extinction coefficient ($\Delta\varepsilon$) at 295 nm.

pH-dependence of the unphosphorylated bovine pepsin A-catalyzed hydrolysis of the hexapeptide

The steady-state kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) are listed in Table I. The logarithm of k_{cat}/K_m was plotted against pH (Fig. 3). Such a plot reflected two apparent pK values: $pK_{e_1} = 1.2$ and $pK_{e_2} = 5.0$ for ionization of two groups of the free enzyme. Activity was maximum around pH 3.0. By initial rate measurements (results not shown) in which whole bovine pepsin A treated or not by phosphatase were compared, optimum activity was observed between pH 3.5 and 3.2 for both enzymes.

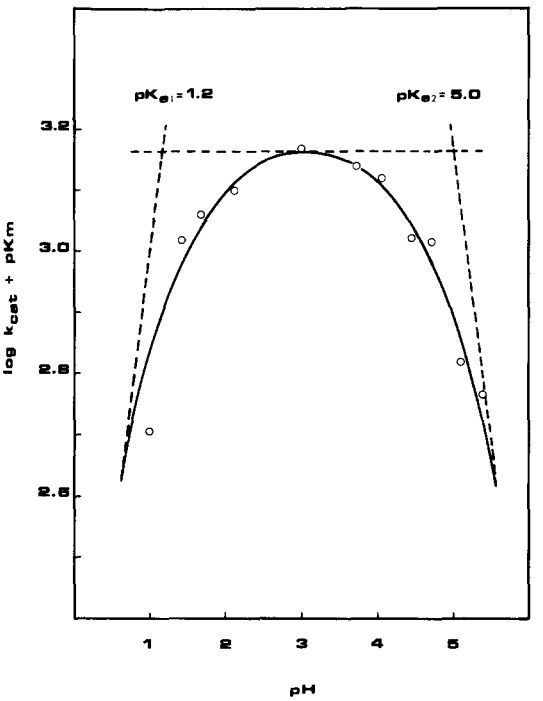

Fig. 3. pH dependence of the unphosphorylated bovine pepsin A-catalyzed hydrolysis of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe.

TABLE I

STEADY-STATE KINETIC PARAMETERS OF THE UNPHOSPHORYLATED BOVINE PEPSIN A-CATALYZED HYDROLYSIS OF Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe

All experiments were carried out at 30°C in 0.1 M sodium citrate buffer. Final pepsin concentration was 1.85 nM. n, number of initial velocities recorded over substrate concentration range (2–3 initial velocities per substrate concentration). Results are given with their 0.95% confidence limits.

pH	λ (nm)	$\Delta\varepsilon_\lambda^{pH}$ ($M^{-1}\cdot cm^{-1}$)	S (μM)	n	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
0.98	295	−235	12.9– 51.6	16	49.3± 8.3	0.097±0.021	508
1.42	295	−215	12.9– 51.6	14	86.0± 6.3	0.082±0.013	1056
1.67	295	−200	12.9– 51.6	12	59.6± 7.4	0.052±0.015	1155
2.12	295	−160	12.9– 51.6	15	109.4± 7.6	0.087±0.016	1257
2.99	295	100	12.9–103.2	12	67.9±10.2	0.053±0.010	1284
	310	375		11	87.7± 6.3	0.053±0.008	1654 } 1468
3.73	310	680	12.9–103.2	14	95.8± 8.1	0.070±0.009	1374
4.06	310	810	12.9–103.2	16	124.0± 6.1	0.094±0.012	1318
4.45	310	940	12.9–103.2	12	139.7± 7.0	0.133±0.013	1050
4.74	310	990	12.9–154.8	10	110.4± 7.7	0.106±0.014	1041
5.10	310	1035	12.9–154.8	11	263.3±12.0	0.401±0.047	657
5.43	310	1060	12.9–154.8	15	603.0±14.4	1.015±0.162	594

Kinetic parameters of the hexapeptide hydrolysis at pH 4.7 by bovine pepsins A with different phosphorylation levels and by bovine gastricsin

Results, obtained from Eisenthal and Cornish-Bowden plots, of which a typical one is shown in Fig. 4, are given in Table II. Phosphorylation level of bovine pepsin A seems to have a relatively weak effect on K_m as well as on k_{cat} values. However, the k_{cat}/K_m ratio observed with the most phosphorylated bovine pepsin A (A_3) treated by phosphatase was half that obtained with unphosphorylated bovine pepsin A though both enzymes were lacking phosphate groups. The kinetic parameters found for bovine gastricsin were closer to those of pepsin than those of chymosin. This was particularly obvious as far as K_m was concerned. The K_m value obtained with gastricsin (0.051 mM) which was of the same order of magnitude as that found for pepsin (ranging between 0.038 and 0.021 mM), was about 20-fold lower than that of chymosin (0.98 mM).

Discussion

From log k_{cat}/K_m vs. pH profile it is reasonable to follow the assumption put forward to propose Scheme 1 according to which two ionizable groups, one with an apparent pK_{e_1} value of about 1.2 and another with pK_{e_2} around 5.0, are involved in the catalytic activity of bovine pepsin A. Though those pK_a values are rough approximation, since experimental data below pH 1 and above pH 5.5 are lacking, thus precluding an accurate drawing of the tangents to the curve, one can suggest that carboxyl groups are concerned. Diazoacetyl-D-L-norleucine methyl ester and 1,2-epoxy-3-(*p*-nitrophenoxy) propane, two active site-directed reagents, were shown to inactivate specifically porcine pepsin by esterifying the two aspartyl groups Asp_{215} and Asp_{32}, respectively [19–21]. Diazoacetyl-DL-norleucine methyl ester stoichiometrically reacts with an aspartic acid residue of bovine pepsin A located in the same heptapeptide se-

TABLE II

STEADY-STATE KINETIC PARAMETERS OF Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe HYDROLYSIS BY BOVINE PEPSIN A WITH DIFFERENT PHOSPHORYLATION LEVELS AND BY BOVINE GASTRICSIN, COMPARED WITH THOSE OF OTHER ASPARTIC PROTEASES

All experiments were carried out at 30°C in 0.1 M sodium acetate buffer, pH 4.7, except (c) which were in 50 mM sodium acetate buffer, pH 4.7. Results are given with their 0.95% confidence limits.

Enzymes	S (mM)	E (nM)	n	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Whole bovine pepsin A	0.011–0.290	0.52	18	83.3 ± 4.7	0.021 ± 0.004	4020
Unphosphorylated bovine pepsin A	0.011–0.290	0.57	18	104.7 ± 3.3	0.024 ± 0.002	4362
Bovine pepsin A_3	0.010–0.200	0.59	17	85.6 ± 6.7	0.031 ± 0.006	2761
Reference bovine pepsin A_3 [a]	0.015–0.260	1.17	17	81.0 ± 5.9	0.033 ± 0.005	2455
Dephosphorylated bovine pepsin A_3 [a]	0.015–0.260	1.17	17	81.3 ± 5.8	0.038 ± 0.009	2140
Bovine gastricsin	0.024–0.263	1.14	15	67.0 ± 7.4	0.051 ± 0.012	1314
Bovine pepsin [b]				54	0.033	1640
Porcine pepsin [b]				100	0.019	5260
Bovine chymosin				13.9	0.81	17.1 [b]
				12.0	0.50	24.0 [c]
				25.1	0.98	25.6 [d]
Mucor pusillus proteinase [d]				3.4	0.083	41.0
Mucor miehei proteinase [d]				5.4	0.130	41.5
Penicillium roqueforti acid proteinase [e]				11.3	0.04	283

[a] The same starting solution of bovine pepsin A_3 was treated (dephosphorylated) or not (reference) by potato acid phosphatase at pH 5.6 during 24 h at 37°C [11]. The reference bovine pepsin A was placed in the same conditions.

[b] Data from Raymond and Bricas [15].

[c] Data from Visser et al. [36].

[d] Data from Martin et al. [7]. k_{cat} and K_m for chymosin are averages of values obtained with chymosins A and B.

[e] Data from Houmard and Raymond [34].

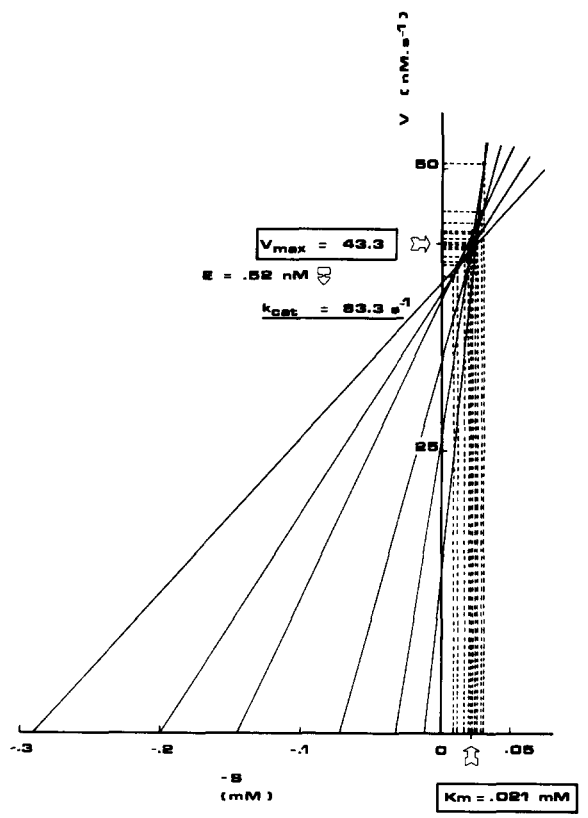

Fig. 4. Typical Eisenthal and Cornish-Bowden direct linear plot for the bovine pepsin A-catalyzed hydrolysis of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe in 0.1 M sodium acetate buffer, pH 4.7, at 30°C.

quence as in porcine pepsin, i.e., Ile-Val-Asp_{215}-Thr-Gly-Thr-Ser [22]. Bovine pepsin A is also inactivated by 1,2-epoxy-3-(*p*-nitrophenoxy) propane (Martin, P., unpublished data). As there is strong structural evidence that the evolutionary highly conservative sequence Ile-Phe-Asp_{32}-Thr-Gly-Ser-Ser is also present in bovine pepsin [23], one can reasonably conclude that the two ionizable groups with $pK_{e_1} = 1.2$ and $pK_{e_2} = 5.0$, our pH-dependence study has revealed, probably correspond to Asp_{32} and Asp_{215}, respectively.

Such kinetic studies do not allow one to define precisely whether the functions played by the ionizable groups thus incriminated are involved in conformational or catalytic events, particularly, because the low substrate solubility precludes explicit measurements of k_{cat} and K_m over a sufficiently wide pH range. The significant increase of both K_m (10-fold) and k_{cat} (6-fold) above pH 5, while their values varied within small limits between pH 1 and 4.7, perfectly illustrates such an alternative. Fruton and co-workers [24–26], working with porcine pepsin, have shown that K_m, estimated from steady-state kinetic measurements using oligopeptide substrates, approximates the value of K_s, the dissociation constant of the enzyme-substrate complex. Further evidence for this conclusion was provided by stopped flow fluorescence measurements [27]. Assuming, k_{cat} and K_m can be considered independently – which is perfectly questionable – and assuming those parameters reflect the catalytic efficiency and the affinity of bovine pepsin A for the hexapeptide, respectively, the results suggest that above pH 5 events occur, affecting simultaneously the binding as well as the catalytic properties. By circular dichroïsm and difference ultraviolet-spectrophotometry it was shown that swine pepsin is subjected to a conformational change above pH 5, coming with modifications in the enzymic properties [28]. As far as k_{cat} is concerned, its increase above pH 5 might imply that catalytic efficiency is significantly improved when the carboxyl group of Asp_{215} is dissociated. This is inconsistent with the generally admitted mechanism of aspartic proteinases. A conformational change, following upon ionization of Asp_{215} or the ionization of a third catalytically important group around pH 5 might explain such a discrepancy. The involvement of a third carboxyl group, in the catalytic mechanism of swine pepsin has already been suggested [21,29]. That presumed third carboxyl group might belong to Asp_{304}. Indeed, Asp_{304} which is located in a highly conservative surrounding [23]: Gly-Asp-Val-Phe-Ile-Arg (or Lys), was associated to a conformational change, induced by substrate binding, through an ion-pair with Lys_{308} (Arg in porcine pepsin) in penicillopepsin [30]. The involvement of an arginine residue in pepsin mechanism has also been postulated [21].

The α-NH_2 of the hexapeptide N-terminal leucine residue, which is protonated in the pH range investigated, might also be involved above pH 5, following upon the ionization of a group belonging to or located near the binding site.

As discussed elsewhere [11], differences observed between dephosphorylated bovine pepsin A_3 and unphosphorylated bovine pepsin A, both

lacking phosphate groups, is likely the consequence of the heterogeneity displayed in isoelectric focusing by bovine pepsin A_3. Indeed, this preparation contained slight amounts of active materials likely coming from limited autolysis (cf. Fig. 5). Bovine pepsin A_3 dephosphorylated or not, exhibits the same kinetic constants at pH 4.7, suggesting that post-translational phosphorylation is without any significant effect on its catalytic activity towards oligopeptides such as Leu-Ser-Phe(NO_2)-Nle-Ale-Leu-OMe, as it was previously shown with dephosphorylated porcine pepsin using a smaller synthetic peptide [14]. However, it seems that the activity of bovine pepsin A towards protein substrates could be affected by its phosphate content [11].

Although the action of porcine pepsin [31] and chymosin [5] on small peptides appears to be independent of ionic strength, buffering species seem to have some influence on steady-state kinetic parameters of bovine pepsin A. Indeed, while k_{cat} remained constant (104.7 vs 110.4 s^{-1}), a 5-fold increase in K_m was observed (0.024 vs. 0.106 mM), when the results of the pH-dependence study performed in citrate buffer at pH 4.7 (see Table I) and those of the study on the influence of phosphate content carried out with unphosphorylated bovine pepsin A in acetate buffer (Table II) are compared. Such an observation, mentioned previously for cationic peptide substrates of porcine pepsin [32], is presumably ascribable to the polycarboxylic nature of citrate.

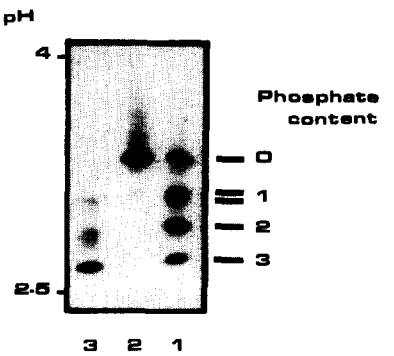

Fig. 5. Isoelectric focusing patterns of whole bovine pepsin A (1) dephosphorylated bovine pepsin A_3 (2) and bovine pepsin A_3 (3). Experimental conditions are detailed elsewhere [11]. Phosphate content is in mole per molecule of pepsin.

Among the synthetic oligopeptides on which bovine pepsin A was tested, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe appears as being the most sensitive one (see Table III). The k_{cat}/K_m ratio obtained with that peptide reaches 4020 $mM^{-1} \cdot s^{-1}$, thus approaching one of the highest value (7050 $mM^{-1} \cdot s^{-1}$) hitherto found for porcine pepsin with the pyridyl tetrapeptide: Z-Ala-Ala-Phe-Phe 0P4P [24]. The new chromogenic hexa- and heptapeptides recently proposed [33] to study zymogen activation, on which hog and chicken pepsins were

TABLE III

KINETIC PARAMETERS OF BOVINE PEPSIN A ACTION ON SYNTHETIC OLIGOPEPTIDES

Kinetic parameters were measured in 3% methanolic, 5 mM sodium phosphate buffer (pH 2.0) at 37.6°C, for AcPhe-Tyr, and in 0.1 M sodium acetate buffer (pH 4.7), at 30°C, for all other peptides.

Peptides	k_{cat} (s)	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe [a]	83.3	0.021	4020
[b]	54.0	0.033	1660
Leu-Ala-Phe(NO_2)-Phe-Ala-Leu-OMe [b]	16.8	0.042	400
Leu-Ser-Phe(NO_2)-Phe-Ala-Leu-OMe [c]	36.7	0.087	423
Phe-Gly-His-Phe(NO_2)-Nle-Ala-Leu-OMe [c]	14.7	0.028	525
Ac Phe -Tyr [d]	0.1	1	

[a] Data from the present study.
[b] Data from Raymond and Bricas [15].
[c] Data from Chaix and Bricas [37].
[d] Data from Lang and Kassell [8].

tested, are far less sensitive.

On the other hand, as outlined in a previous paper [7], aspartic proteinases so far tested on Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe may be divided into two classes according to their catalytic efficiency (i.e., k_{cat}/K_m ratio). Bovine gastricsin and pepsins A, whatever the phosphate content of the latter is, belong to the first one, including also porcine pepsin, while the second class gathers chymosin and *Mucor* aspartic proteinases. Aspartic proteinase from *Penicillium roqueforti*, tested by Houmard and Raymond [34] occupies an intermediate position ($k_{cat}/K_m = 283\ mM^{-1} \cdot s^{-1}$) with a catalytic rate constant (k_{cat}) close to those of the enzymes belonging to the second class and a K_m close to those of the enzymes belonging to the first. It must be quoted that *P. roqueforti* proteinase shows a wider primary specificity and that, as penicillopepsin, aspartic proteinases from *Endothia parasitica* and *Rhizopus chinensis*, it is able to activate trypsinogen by cleaving a Lys-Ile peptide bond. Taking advantage of this feature, a sensitive chromophoric hexapeptide, in which a Lys-Phe(NO_2) bond is cleaved, has been proposed for fungal aspartic proteinases exhibiting a trypsinogen-activating ability [35].

Note added in proof (Received October 8th, 1984)

Dunn et al. [38] have described recently the synthesis and evaluation of a new chromophoric substrate for aspartic proteinases whose design is based on subsite preferences. That substrate, the heptapeptide Pro-Thr-Glu-Phe↓Phe(NO_2)-Arg-Leu, appears to be less sensitive than the hexapeptide used in the present paper. Values of k_{cat}, K_m and k_{cat}/K_m of 94 s^{-1}, 0.13 mM and 815 $mM^{-1} \cdot s^{-1}$ have been obtained at pH 3.0 and 37°C. Nevertheless, the stability of $\Delta\xi$ and solubility of the peptide over the pH range 2–5 are interesting features, especially as far as determination of the pH-dependence of the kinetic parameters is concerned.

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