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Fragmentation of proteins by *S. aureus* strain V8 protease

Ammonium bicarbonate strongly inhibits the enzyme but does not improve the selectivity for glutamic acid

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Staphylococcus aureus strain V8 protease is a serine endopeptidase which cleaves peptide bonds at the carboxyl side of Glu and Asp. Specific cleavage at Glu has previously been achieved in ammonium bicarbonate whereas in sodium phosphate cleavage at both Glu and Asp was observed. However, it is shown here that bicarbonate does not restrict the specificity to Glu-X bonds, it simply inhibits the enzyme. The degradation of a mixture of oxidized insulin and glucagon proceeds similarly in the two buffers, although faster in phosphate.

Staphylococcus aureus; Protease; Serine endopeptidase; Specificity; Amino acid sequence analysis

1. INTRODUCTION

A serine endopeptidase from *Staphylococcus aureus* strain V8, first isolated by G.R. Drapeau [1] is widely used for fragmentation of proteins, prior to amino acid sequence determinations, due to its specificity for Glu-X and Asp-X peptide bonds [2–4]. Earlier investigations [2,3] have indicated that the specificity of this endopeptidase is dependent on the buffer: in ammonium bicarbonate (pH 7.8) or in ammonium acetate (pH 4.0) only Glu-X bonds were cleaved while in sodium phosphate (pH 7.8) Glu-X as well as Asp-X bonds were hydrolyzed. Furthermore, monovalent anions have been shown to inhibit the *S. aureus* strain V8 protease [5].

In the present communication, it is shown that the enzyme cleaves approximately 3000-fold faster at Glu as compared to Asp in otherwise identical synthetic substrates, essentially independent of the buffer. The effect of ammonium bicarbonate and acetate is only inhibitory.

2. MATERIALS AND METHODS

2.1. *Materials*

Staphylococcus aureus strain V8 protease was from ICN Biomedicals Inc., Illinois, USA. The internally quenched fluorescent substrates ABz-Ala-Phe-Ala-Phe-X-Val-Phe-Tyr(NO_2)-Asp-OH where X = Asp or Glu was a gift from Dr. Morten Meldal, Carlsberg Laboratory. Porcine glucagon and insulin was a gift from NOVO NORDISK, Denmark. Insulin was oxidized by performic acid as described by Hirs [6], lyophilized and the mixture used directly for the digestion studies.

Abbreviations: ABz, *o*-aminobenzoyl (anthraniloyl); CAPS, 3-(cyclohexylamino)propanesulphonic acid; CHES, 2-(cyclohexylamino)ethanesulphonic acid; TFA, trifluoroacetic acid.

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2.2 *Enzyme assays*

Enzymatic hydrolysis of the intramolecularly quenched substrates was performed as previously described [7]. The substrates were dissolved in dimethylformamide and 50 μl was added to 2450 μl buffer solution, and the initial fluorescence measured. V8 protease, dissolved in water, was added. The emission at 420 nm upon excitation at 320 nm was followed with time until hydrolysis was complete. k_{cat}/K_m was determined from initial rates using the relation $k_{cat}/K_m = V_o/(S_o E_o)$ which is valid at $S_o << K_m$. The value listed is an average of a minimum of two values varying less than 5%, obtained at two substrate concentrations, typically around 0.1 μM and 0.2 μM, to ensure the validity of the equation. The influence of anions on the activity of V8 protease was followed using the synthetic substrates in 50 mM HEPES and different concentrations of anions, pH 7.8. The pH-dependence of the activity was similarly investigated using 20 mM buffer, 0.1 M NaCl. The following buffers were used: formic acid pH 3.4–4.2; acetic acid, pH 4.2–5.5; MES, pH 5.5–6.8; HEPES, pH 6.8–7.7; BICINE, pH 7.7–8.7; CHES, pH 8.7–9.4; CAPS, pH 9.4–10.5.

Enzymatic hydrolysis of glucagon and oxidized insulin was followed by HPLC. The peptides were dissolved in 6 M urea and diluted to 2 M urea with buffer to final substrate concentrations of 0.4 mM each. The buffer was either sodium phosphate or ammonium bicarbonate, containing EDTA. Enzyme, dissolved in water, was added at concentrations of 0.001 mg/ml or 0.2 mg/ml. The buffers were either 30 mM sodium phosphate, 1.2 M urea, 2 mM EDTA, pH 7.8 or 60 mM ammonium bicarbonate, 1.2 M urea, 2 mM EDTA, pH 7.8. Digestion was followed with time and the hydrolysis products separated on a reverse phase C_{18}-column from Vydac employing a 50 min acetonitrile gradient from 5 to 45% in 0.1% TFA. Cleavage products were identified by amino acid analysis after hydrolysis in 6 N HCl or N-terminal amino acid sequence determinations.

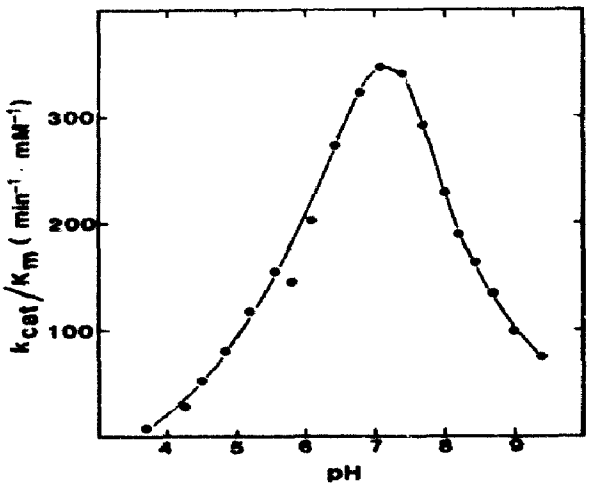

Fig. 1. pH-dependence of the hydrolysis of ABz-Ala-Phe-Ala-Phe-Glu-Val-Phe-Tyr(NO_2)-Asp-OH by *S. aureus* strain V8 protease. For further details see section 2.2.

3. RESULTS AND DISCUSSION

The pH-dependence of the hydrolysis of ABz-Ala-Phe-Ala-Phe-Glu-Val-Phe-Tyr(NO_2)-Asp-OH by *S. aureus* strain V8 protease was bell-shaped with an optimum at pH 7.2 (Fig. 1), apparently dependent on two ionizable groups in the enzyme with pK_a values of 5.8 and 8.4. This is in contrast to earlier investigations with hemoglobin as substrate: two pH-optima at pH 4.0 and pH 7.8 [1] were found and this could be due to an effect of pH on the hemoglobin substrate. For the subsequent studies a pH of 7.8 was chosen to allow comparisons with earlier investigations in the literature.

Earlier reports [2,5] have indicated that *S. aureus* strain V8 protease is inhibited by monovalent anions. This inhibition by chloride, acetate and nitrate was confirmed using ABz-Ala-Phe-Ala-Phe-Glu-Val-Phe-Tyr(NO_2)-Asp-OH as substrate and increasing concentrations of anions (Fig. 2B). Presumably, the inhibition is partially due to ionic interactions being important for the interaction between enzyme and substrate and such interactions are adversely affected by increasing ionic strength. However, in addition it was found that phosphate and sulfate had an activating effect on the enzyme (Fig. 2A) which may be ascribed to the tetrahedral configuration of these anions as compared to the planar configuration of the inhibitory acetate, borate, nitrate and bicarbonate. A specific interaction of phosphate and sulfate with the enzyme is indicated.

The hydrolysis of ABz-Ala-Phe-Ala-Phe-X-Val-Phe-Tyr(NO_2)-Asp-OH (X = Glu or Asp) in different buffers revealed (Table I) that the substrate with X = Glu is hydrolyzed 3000–5000-times faster than that with X = Asp with only little dependence of this ratio on the nature of the buffer.

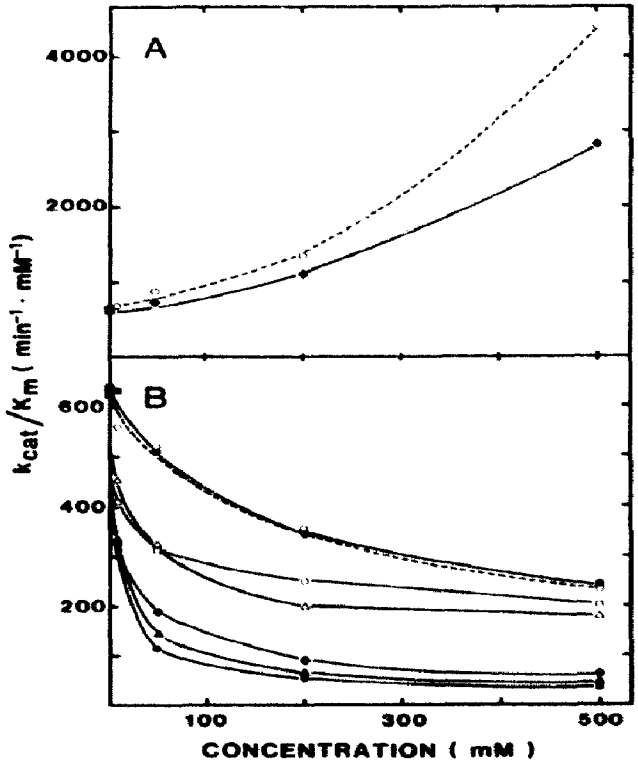

Fig. 2. The influence of anions on the hydrolysis of ABz-Ala-Phe-Ala-Phe-Glu-Val-Phe-Tyr(NO_2)-Asp-OH by *S. aureaus* strain V8 protease at pH 7.8. (A) Activation by sodium phosphate (○) and Na_2SO_4 (●). (B) Inhibition by NaCl (■), NH_4Cl (□), sodium acetate (▽), borate (△), $NaNO_3$ (◆), NH_4HCO_3 (▲) and $NaHCO_3$ (●). For further details see section 2.2.

To extend these investigations to larger substrates, the initial cleavage of the B-chain of oxidized insulin (Glu^{13}-Ala^{14}) in phosphate and bicarbonate buffer, respectively, was followed by HPLC at low enzyme concentration (0.001 mg/ml). From the peak area of the B-chain the amount cleaved per minute was calculated and it was found that the rate of cleavage was approxi-

Table I

Hydrolysis of ABz-Ala-Phe-Ala-Phe-X-Val-Phe-Tyr(NO_2)-Asp-OH where X = Glu or Asp by *S. aureus* strain V8 protease in different buffers

Buffer	k_{cat}/K_m for substrate where X = Glu / k_{cat}/K_m for substrate where X = Asp
50 mM HEPES, pH 7.8	3100
50 mM HEPES, 2 M urea, pH 7.8	4100
0.1 M sodium phosphate, pH 7.8	4300
0.1 M ammonium bicarbonate, pH 7.8	5300

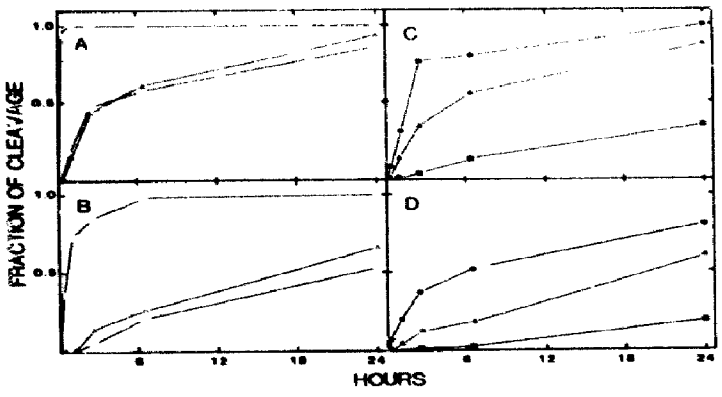

Fig. 3. Digestion of a mixture of oxidized porcine insulin and porcine glucagon in sodium phosphate with enzyme concentrations of 0.001 mg/ml (A) and 0.2 mg/ml (C) or in ammonium bicarbonate with enzyme concentrations of 0.001 mg/ml (B) and 0.2 mg/ml (D). Cleavage of Glu^{13}-Ala^{14} (B-chain) (○), Glu^{21}-Arg^{22} (B-chain) (△), Glu^{17}-Asn^{18} (A-chain) (□), Asp^{15}-Ser^{16} (●), Asp^{21}-Phe^{22} (▲) and Asp^{9}-Tyr^{10} (■).

mately ten-times faster in phosphate (0.030 mM/min) as compared to bicarbonate (0.0028 mM/min).

To further investigate the selectivity of *S. aureus* strain V8 protease for Glu-X bonds, performic acid oxidized insulin and glucagon were digested in phosphate and bicarbonate buffer at high and low enzyme concentrations. Insulin contains no Asp-X bonds but the following Glu-X bonds: Glu^{4}-Gln^{5} and Glu^{17}-Asn^{18} in the A-chain, Glu^{13}-Ala^{14} and Glu^{21}-Arg^{22} in the B-chain. Glucagon contains no Glu-X bonds but the following Asp-X bonds: Asp^{9}-Tyr^{10}, Asp^{15}-Ser^{16} and Asp^{21}-Phe^{22}. In phosphate, at low enzyme concentration (0.001 mg/ml), the Glu^{13}-Ala^{14} (B-chain) bond was cleaved very quickly (Fig. 3A) whereas Glu^{17}-Asn^{18} (A-chain) and Glu^{21}-Arg^{22} (B-chain) were cleaved at a much lower rate (Fig. 3A). Glu^{4}-Gln^{5} (A-chain) and peptide bonds involving aspartic acid were not cleaved (not shown). In bicarbonate, at low enzyme concentration, the same peptide bonds were cleaved but at lower rates (Fig. 3B).

At high enzyme concentration (0.2 mg/ml), the peptide bonds involving Glu were cleaved almost instantaneously except Glu^{4}-Gln^{5} (A-chain) which was cleaved slowly (data not shown). The Asp-X bonds were cleaved at much lower rates. In phosphate buffer in the following decreasing order (Fig. 3C): Asp^{15}-Ser^{16} > Asp^{21}-Phe^{22} > Asp^{9}-Tyr^{10}. In bicarbonate the rates were even lower, but the pattern was the same (Fig. 3D). Thus, the Asp-X bonds are also cleaved in ammonium bicarbonate although at lower rates than in sodium phosphate, but this is also the case for the Glu-X bonds. Consequently, the preference for Glu over Asp is not significantly affected by the nature of the buffer as previously suggested [2,3]. The beneficial effects previously observed are probably due to the extensive inhibition of the enzyme by bicarbonate which reduces its action to the preferred Glu-X bonds.

In conclusion, the procedures for the widespread use of the *S. aureus* strain V8 protease for generating peptides from large proteins for amino acid sequence studies should be modified. Cleavage in ammonium bicarbonate at a high enzyme-to-substrate ratio (1:30 w/w) should be abandoned in favour of a much lower enzyme concentration in phosphate buffer.

REFERENCES

[1] Drapeau, G.R., Boily, Y. and Houmard, J. (1972) J. Biol. Chem. 247, 6720–6726.
[2] Houmard, J. and Drapeau, G.R. (1972) Proc. Natl. Acad. Sci. USA 69, 3506–3509.
[3] Drapeau, G.R. (1977) Methods Enzymol. 47, 189–191.
[4] Austen, B.M. and Smith, E.L. (1976) Biochem. Biophys. Res. Commun. 72, 411–417.
[5] Houmard, J. (1976) Eur. J. Biochem. 68, 621–627.
[6] Hirs, C.H.W. (1967) Methods Enzymol. 11, 59–62.
[7] Meldal, H. and Breddam, K. (1991) Anal. Biochem. 195, 141–147.