

THE HYDROLYSIS OF THE THREE CLUPEIN COMPONENTS BY SUBTILISIN NOVO, SUBTILISIN CARLSBERG AND NITRATED SUBTILISIN CARLSBERG

by

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The three components of clupein (Y^I, Y^{II} and Z) were hydrolyzed by subtilisin Carlsberg and subtilisin Novo. Subtilisin Novo hydrolyzed all three components much faster than did subtilisin Carlsberg, but components Y^{II} and Z were hydrolyzed with the same initial rate and faster than component Y^I. The primary bonds cleaved in component Y^{II} were Ser₂₂-Arg₂₃ followed by Ala₈-Ser₉. Ser₂₁-Arg₂₂ followed by Ala₉-Ser₁₀ were the primary bonds cleaved in clupein-Z. It was further demonstrated that nitrated subtilisin Carlsberg had become more specific for cleavage of the Ser-Arg bond compared with the unmodified enzyme. With clupein Y^I as the substrate, the peptide pattern obtained was too complicated for a particular bond to be identified as the primary attacking point for the two unmodified enzymes. Nitrated subtilisin Carlsberg attacked initially the bonds Arg₃-Ser₆ and Ser₆-Ser₇.

1. INTRODUCTION

Clupein sulfate (a protamine isolated from herring sperms) is a good substrate for subtilisin Novo (10). Subtilisin Carlsberg degrades this substrate 8 to 10 times slower, primarily due to weaker binding (11). However, nitration of

Tyr₁₀₄ in subtilisin Carlsberg increases the rate of hydrolysis of clupein sulfate to the same level as with subtilisin Novo (17). This finding was explained by the introduction of a negative charge in a secondary binding site, S₄¹⁾, on subtilisin Carlsberg, a site which most likely will contain

1. The subsite nomenclature of SCHECHTER and BERGER (14) has been adopted in the present paper for the description of the binding of substrates to the subtilisins.

Tyr₁₀₄. KRAUT and coworkers (13) have shown from X-ray crystallographic studies that Tyr₁₀₄ was part of subsite S₄ in subtilisin BPN' (identical to subtilisin Novo). Although the detailed three-dimensional structure of the Carlsberg enzyme has yet to be published, it is almost certain that Tyr₁₀₄ also is part of S₄ in this enzyme since the two enzymes are highly homologous (20). A model of subtilisin Carlsberg built in our laboratory on the basis of the coordinates for subtilisin BPN' is also compatible with Tyr₁₀₄ being part of subsite S₄.

The objects of the present study were to locate the bonds initially cleaved in clupein by subtilisin Carlsberg and subtilisin Novo, and to determine if the same bonds are hydrolyzed by nitrated subtilisin Carlsberg. Since the sequences of the three components of clupein (Y^I, Y^{II} and Z³) are known (1) it is possible to compare the results with the three-dimensional models of the subtilisins, and to check if they are compatible with the binding mode for peptides proposed by KRAUT and coworkers (13). Since clupein does not contain any of the amino acid residues Tyr, Leu or Phe, which from the studies of MORIHARA and coworkers (7) are expected to bind preferentially in S₁, this particular polypeptide is well suited for the study of the secondary binding sites in the subtilisins.

Finally, the rate of hydrolysis of the individual clupein components has been studied in order to determine if one of the components was hydrolyzed much faster than the two others and consequently would determine the initial rate of hydrolysis of unfractionated clupein.

2. MATERIALS AND METHODS

2.1 Materials

Subtilisin Novo (Batch 120-4) and subtilisin Carlsberg (Batch 70-3) were gifts from Novo Industries, Copenhagen, Denmark. Nitrated subtilisin Carlsberg containing 5.8 moles of nitrotyrosine per mole of enzyme was prepared as described by SVENDSEN (17).

2.2 Preparation of the individual clupein components

Clupein sulfate was prepared from dried herring sperms essentially as described by SCHMIEDEBERG (15). This preparation consists of three polypeptide chains in almost equal amounts but with different amino acid composition. Further separation into the individual chains was achieved by the method of ANDO (2), scaled up to handle 2 g quantities. A Biogel CM-2 column (2.6 × 100 cm) was equilibrated with a 0.05 M-Na-acetate buffer, pH 5.8, containing 1.5 M of NaCl and 0.02% NaN₃. 2 g clupein sulfate was dissolved in 50 ml of the same buffer and centrifuged to remove a small amount of undissolved material. The clear supernatant was applied on the top of the column, and the elution performed with the same buffer. The flow-rate was 14.5 ml/hr, and fractions of 10 ml were collected. The fractionation was made at room temperature due to the poor solubility of clupein sulfate at 4°. The three clupein containing peaks were located by the Sakaguchi reaction (5), pooled individually, dialyzed extensively against distilled water and lyophilized. Although the molecular weight of clupein is only about 4000 daltons, complete recovery of material in the dialysis step was achieved. The isolated components were obtained as the chloride salts. These are very hygroscopic, as opposed to clupein sulfate, and were stored in a vacuum desiccator.

2.3 Kinetic experiments

Kinetic experiments were conducted in a pH-stat (Radiometer titrator TTT2, equipped with a syringe burette, type SBU 10). Two ml of clupein (0.1-0.5%) was brought to 30° in the water-jacketed pH-stat vessel and a suitable amount of enzyme added in a small volume (25 or 50 µl). The base-uptake (0.02 M-NaOH) was recorded on a Radiometer Titrigraph SBR2b and the initial rate of reaction calculated from the slope of the tangent to the curve through the starting point.

2. The nomenclature of ANDO (1) has been used to describe the three clupein components.

2.4 Separation of hydrolytic peptides

Two ml of a 0.5% solution of clupein Y^I, Y^{II} or Z were hydrolyzed in the pH-stat by subtilisin Carlsberg, nitrated subtilisin Carlsberg or subtilisin Novo until an uptake of 2 μ eq. NaOH had been recorded. HCl (1 M) was added, quickly lowering the pH to about two, and the solution was left for 10 min to denature the enzyme. The solution was then transferred to a flask, lyophilized and redissolved in 1 ml of a 0.05 M-Na-acetate buffer, pH 5.8. 50 μ l were withdrawn for amino acid analysis to determine the amount subsequently applied to the ion-exchange column. This was carboxymethyl cellulose (0.9 \times 52 cm, Whatman CM 52), equilibrated with a 0.05 M-Na-acetate buffer, pH 5.8. Elution of the peptides was done at room temperature with a linear gradient made from 110 ml 0.05 M-Na-acetate, pH 5.8 + 0.5 M-NaCl in the first chamber, and 110 ml 0.05 M-Na-acetate, pH 5.8 + 1.5 M-NaCl in the second chamber of the gradient mixer. Both buffers contained 0.02% NaN₃. The flow-rate was 15 ml/hr and fractions of 2.5 ml were collected. The eluted peaks were located by the Sakaguchi reaction (5). The individual peaks were pooled and after thorough mixing a suitable aliquot was withdrawn for amino acid analysis.

2.5 Amino acid analysis

Hydrolyses were made in constant boiling HCl at 110° for 24 hrs in sealed evacuated tubes. The hydrolyzed samples were dried in a 40° water-bath under a stream of air. The amino acid analyses were done on an amino acid analyzer, Durrum Model 500, according to the procedure given by the manufacturer.

3. RESULTS

3.1 Separation of clupein components

As shown in Fig. 1 a good separation of the three components in clupein sulfate, prepared according to SCHMIEDEBERG (15), was achieved. The components emerged in the following order: Y^{II}, Y^I and Z. Since the clupein reported by ANDO was isolated from the Pacific herring and not the Atlantic herring, as was the case in the present study, the possibility of small

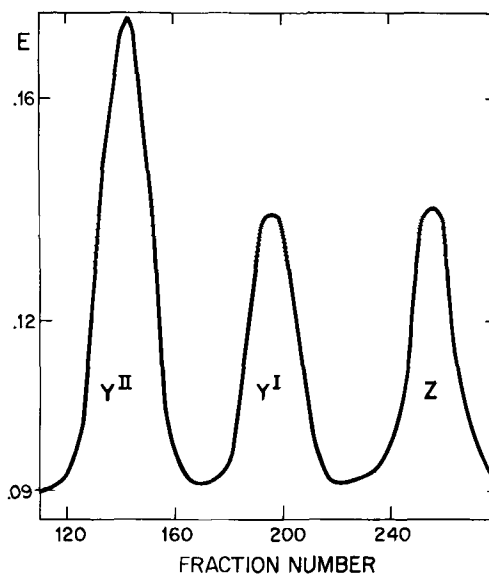


Figure 1. Fractionation of clupein on Biogel CM-2 (2.6 \times 100 cm). Elution buffer: Na-acetate (0.05 M) pH 5.8 + 1.5 M-NaCl + 0.02% NaN₃, Flow-rate: 15 ml/hr. Fractions of 5 ml were collected. Room-temperature. Ordinate: E_{520 nm}, abscisse: fraction number.

differences in amino acid composition exists. However, the amino acid analyses on the three components agreed well with those reported by ANDO. Differences in the sequences cannot be finally excluded before sequence determinations have been performed on the clupeins from the Atlantic herring, but in those cases where clean peptides were obtained after hydrolysis with the subtilisins no difficulty was found in fitting the amino acid composition to fragments of clupein according to the sequence given by ANDO. The interpretation of the results in the present paper rests on the assumption that identity in sequence exists between the clupeins isolated from the Pacific and the Atlantic herring, respectively.

3.2 Kinetic studies

The rate of hydrolysis of increasing concentrations of clupein Y^I, Y^{II} and Z by subtilisin Carlsberg and subtilisin Novo was studied. It has been shown previously (11) that addition of 0.1 M-NaCl to the medium has only a minor effect on the hydrolysis of unfractionated clupein sulfate, and the first series of ex-

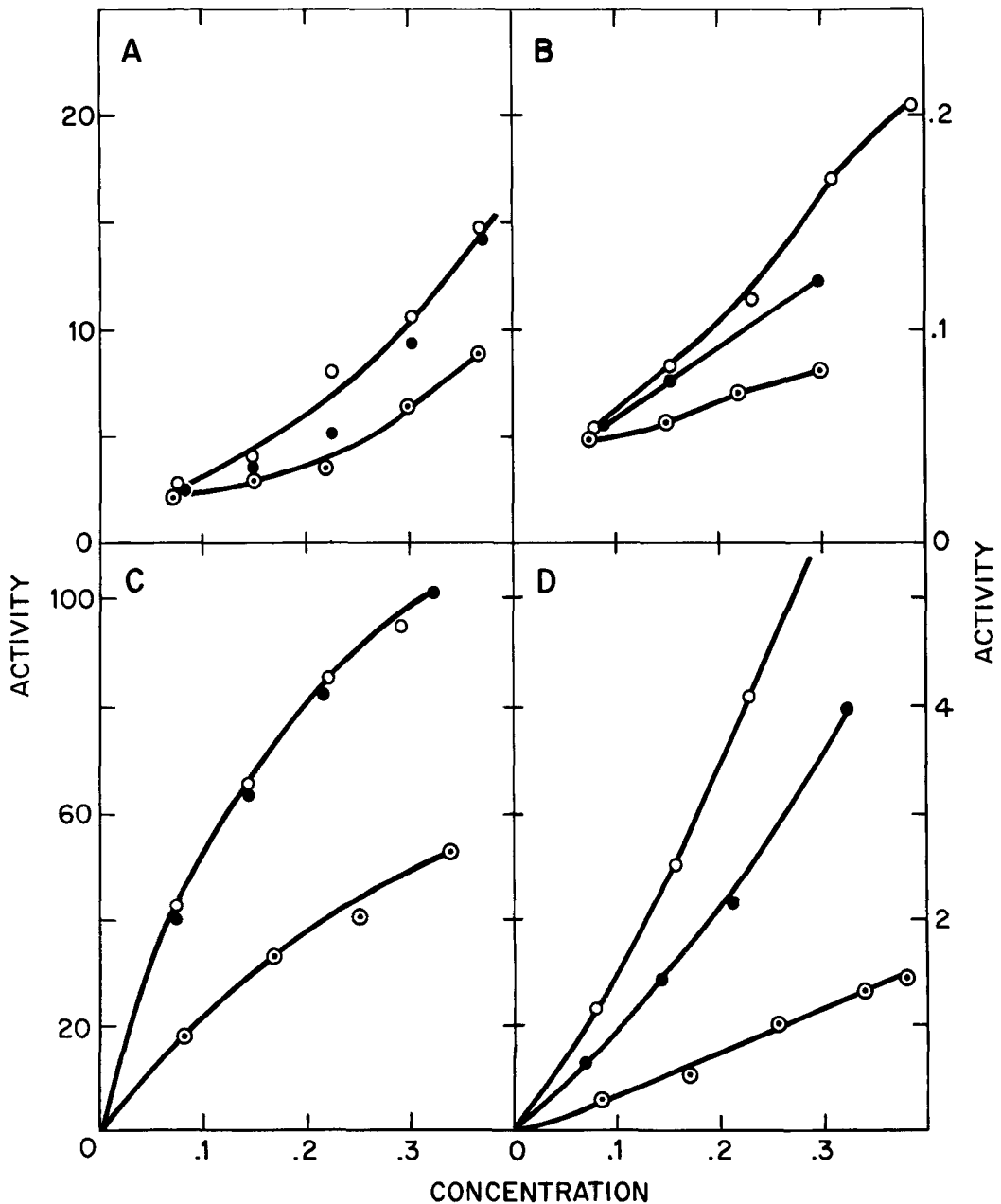


Figure 2. The hydrolysis of clupein YI (—○—), YII (—○—) and Z (—●—) by subtilisin Novo and subtilisin Carlsberg. Abscisse: clupein concentration in per cent. Ordinate: rate of hydrolysis expressed as $\mu\text{eq. H}^+/\text{min}/\text{mg enzyme}/\text{ml}$ in a 2 ml reaction mixture, at Ph 8.0 and 30°. A and C: digestion with subtilisin Novo in distilled water (A) and 0.1 M-NaCl (C). B and D: digestion with subtilisin Carlsberg in distilled water (B) and 0.1 M-NaCl (D).

periments with the fractionated clupein chlorides was accordingly carried out in distilled water. However, the observed rates of hydrolysis, Fig. 2 (A and B) were much smaller than would be expected from the earlier experiments with unfractionated clupein sulfate, although the marked difference in efficiency of the two enzymes persisted, subtilisin Novo being the more effective. Clupein YII and Z were

hydrolyzed with identical rates by subtilisin Novo. Since the amino acid sequences of these clupeins are nearly identical, the result suggested that the initial attack by the enzyme took place in the part of the polypeptide which was identical in the two components. Y^I was hydrolyzed with a slower rate than the two other components. Similar results were obtained with subtilisin Carlsberg. Y^I was hydrolyzed with the slowest rate, but the Z-component was also hydrolyzed more slowly than Y^{II} (Fig. 2 (A and B)).

Since these experiments were performed with clupein chlorides rather than sulfates, they were repeated with 0.1 M-NaCl added to the medium to test if the slow reaction-rate observed without added salt originated from electrostatic repulsions. The screening effect of 0.1 M-NaCl is demonstrated in Fig. 2 (C and D). The rate of hydrolysis of Y^{II} and Z increased to the same level as found with unfractionated clupein sulfate, and the shapes of the curves also changed (compare with Fig. 2 (A and B)). $K_M(\text{app})$, calculated for the subtilisin Novo- Y^{II} or -Z complex from a Lineweaver-Burk plot, was 7×10^{-4} M in good agreement with 8×10^{-4} M calculated for clupein sulfate (11). In this case, Y^I was also hydrolyzed slower than the two other components by both enzymes, and on a relative basis the enhancement of rate was less than for components Y^{II} and Z.

That the nature of the counter-ion determined whether extra salt had to be added in order to maintain the usual rate of hydrolysis was demonstrated by two additional experiments. In the first experiment, unfractionated clupein sulfate was dialyzed against NaCl followed by dialysis against deionized water. The rate of hydrolysis decreased to the level shown in Fig. 2 (A and B). In the second experiment, the fractionated clupeins were dialyzed against Na_2SO_4 (0.1 M) followed by dialysis against deionized water³). The rate of hydrolysis was now increased to the level obtained with clupein chloride + 0.1 M-NaCl.

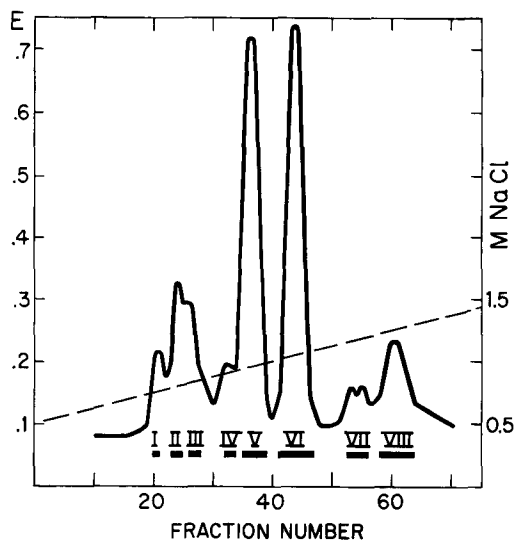


Figure 3. Chromatography of clupein Y^{II} , digested with subtilisin Novo, on Whatman CM 52 (1 x 60 cm). Elution buffers: 110 ml 0.05 M-acetate buffer, pH 5.8 + 0.5 M-CaCl and 110 ml 0.05 M-acetate buffer pH 5.8 + 1.5 M-NaCl. Both buffers contained 0.02% NaN_3 . Fractions of 2.5 ml were collected. Flow-rate 15 ml/hr. Room-temperature.

----- salt-gradient. — fractions pooled for further analysis. Left ordinate: $E_{520 \text{ nm}}$, right ordinate: NaCl conc. (M), abscisse: fraction number.

3.3 Identification of hydrolyzed bonds

3.3.1 Clupein Y^{II}

When clupein Y^{II} was digested with subtilisin Novo and subjected to ion-exchange chromatography, the elution pattern shown in Fig. 3 was obtained. Of the 8 recognizable peaks I, IV and VII were not identifiable with any part of the Y^{II} chain. However, on the basis of amino acid recovery it was calculated that the three unidentified peaks only amounted to 8-9% of the total recovery of amino acids. Since the purpose of this part of the study was to determine the initial bonds attacked by the subtilisins, no further attempts were made to identify the peaks which contributed only little to the overall yield.

As expected from chromatography of unhydrolyzed Y^{II} , peak VIII consisted of Y^{II} . Two peaks, V and VI, were identified as $\text{Pro}_1\text{-Ser}_{21}$

3. In this case the dialysis bag became partly penetratable to clupein. Total recovery, however, was observed when clupein sulfate was dialyzed against NaCl. In both cases the concentration was estimated from amino acid analysis.

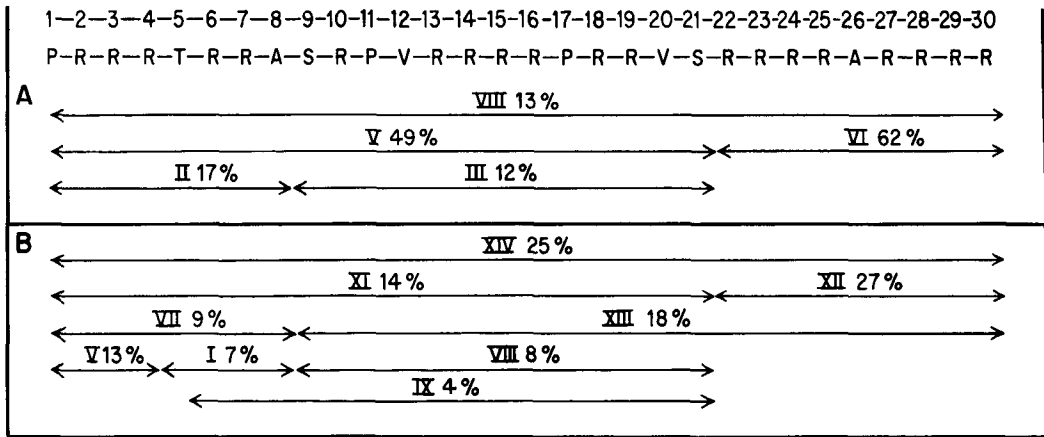
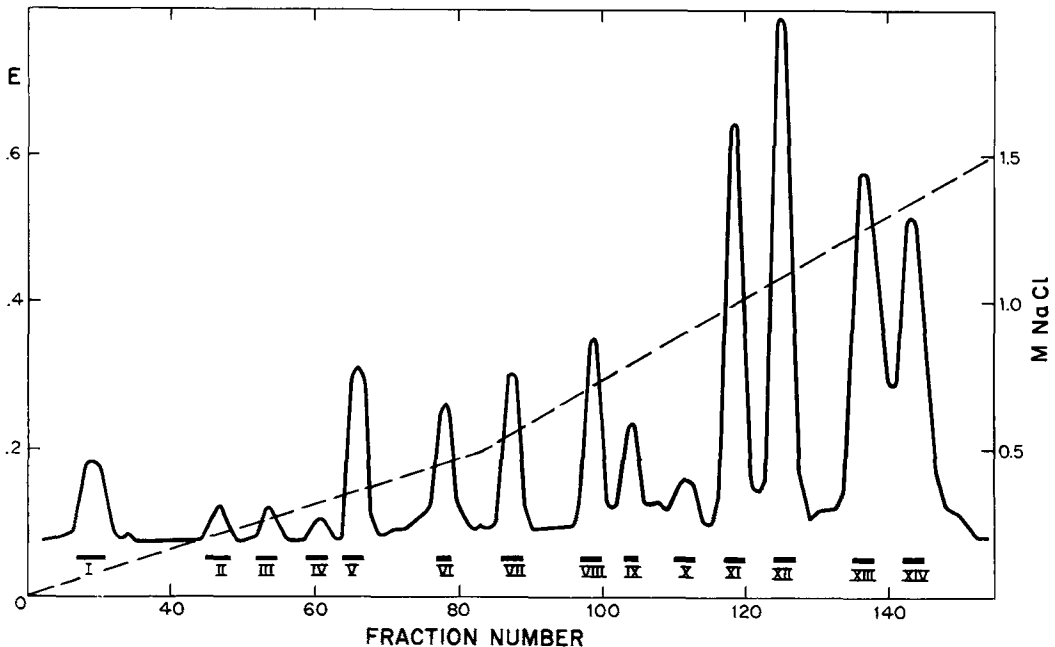


Figure 4. Compositions and yields (In per cent of material applied to the Whatman CM-52 column) of the peptide fragments from digest of clupein YII by A) subtilisin Novo, B) subtilisin Carlsberg. T=Thr, S=Ser, P=Pro, G=Gly, A=Ala, V=Val, I=Ile and R=Arg.

and Arg₂₂-Arg₃₀, respectively, as indicated in Fig. 4, A. Peak II was found to be Pro₁-Ala₈, and peak III was Ser₉-Ser₂₁. The per cent recovery of hydrolytic peptides based on the amount of digest applied to the column is shown in Fig. 4, A. The bond Ser₂₁-Arg₂₂ is the first one to be hydrolyzed by subtilisin Novo, followed by Ala₈-Ser₉.

When YII was hydrolyzed by subtilisin Carlsberg, a chromatogram similar to the one shown in Fig. 3 was obtained after ion-exchange chromatography. However, several overlapping small peaks were located between

Figure 5. Chromatography of clupein YII, digested with subtilisin Carlsberg, on Whatman CM52 (1 × 60 cm). Elution buffer 0.05 M-acetate buffer, pH 5.8, 0.02% NaN₃. Salt gradients: 1) 0-0.5 M-NaCl (220 ml) 2) 0.5-1.5 M-NaCl (220 ml). Fractions: 2.5 ml. Flow-rate: 15 ml/hr. Left ordinate: E_{520 nm}, right ordinate: NaCl conc. (M). Abscisse: fraction number. - - - salt gradient. — Fractions pooled for further analysis.



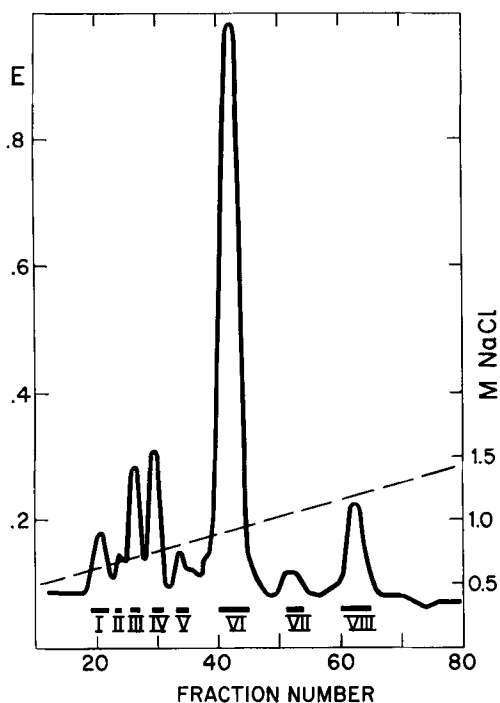


Figure 6. Chromatography of clupein Z, digested with subtilisin Novo, on Whatman CM 52 (1 × 60 cm). Same conditions as in Figure C.

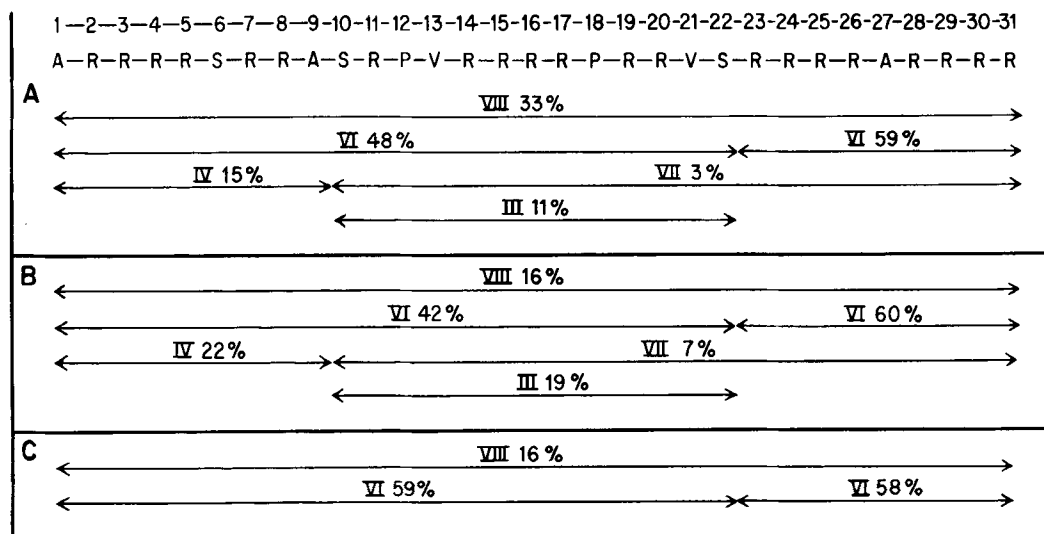
peaks I and IV. In order to obtain a better separation of these peaks, the elution was started with a salt gradient ranging from 0 to 0.5 M-NaCl followed by the usual gradient ranging from 0.5 to 1.5 M-NaCl. The resulting elution pattern is shown in Fig. 5.

The majority of the peaks were identified with known sequences, and their position in the chromatogram together with the yields in which they were isolated are shown in Fig. 4, B. It is seen that the bonds Ser₂₁-Arg₂₂ and Ala₈-Ser₉ are hydrolyzed with almost equal rates in contrast to what was observed with subtilisin Novo. In addition, the bond Arg₄-Thr₅ is hydrolyzed quite readily.

3.3.2 Clupein Z

Because of the close similarity in sequence between the Z (see Fig. 7) and Y^{II} components of clupein, and since they were hydrolyzed with identical rates by subtilisin Novo, it was anticipated that the primary bonds to be hydrolyzed were also identical. This was confirmed by separation of hydrolytic peptides by ion-exchange chromatography, as shown in Fig. 6. As suspected, peak VI with the very high color-yield contained both Ala₁-Ser₂₂ and Arg₂₃-Arg₃₁. This was demonstrated by amino acid analysis of fractions from the ascending and descending limb, respectively, and by gelfiltration on Sephadex G25, whereby the two peptides were separated, followed by amino

Figure 7. Compositions and yields (in per cent of material applied to the Whatman CM 52 column) of peptide fragments from digests of clupein-Z with A) subtilisin Novo, B) subtilisin Carlsberg, C) nitrated subtilisin Carlsberg.



acid analysis. The unidentified peaks (I, II and V) accounted for less than 10% of recovered material.

The yields and peak number of assigned peptides are shown in Fig. 7, A. As expected, the bond Ser₂₂-Arg₂₃ was the one most readily hydrolyzed followed by Ala₉-Ser₁₀.

Chromatography of the hydrolytic peptides from clupein-Z digested by subtilisin Carlsberg showed an elution pattern very similar to the one shown in Fig. 6. Also in this case, unidentified peaks contained less than 10% of the total material eluted. Fig. 7, B gives the yields and peak number of the assigned peptides. The bond Ser₂₂-Arg₂₃ is the most readily hydrolyzed followed by Ala₉-Ser₁₀. At 60% cleavage of the bond Ser₂₂-Arg₂₃ a 26% cleavage was observed at Ala₉-Ser₁₀ with subtilisin Carlsberg, but only 15% with subtilisin Novo. Therefore, it appears that the bond Ala₉-Ser₁₀ is hydrolyzed faster by subtilisin Carlsberg than Novo relative to the bond cleavage at Ser₂₂-Arg₂₃.

3.3.3 Clupein Y^I

No final conclusions can be drawn with respect to the bonds preferentially hydrolyzed in clupein Y^I by the subtilisins. Although well-separated, bell-shaped peaks were eluted from the ion-exchange column with the gradient described in the method section, they appeared to be mixtures of peptides. Using a gradient ranging from 0 to 1.5 M-NaCl the elution pattern showed that several minor components were present. In this case it was possible to identify some of the liberated peptides, and the following bonds were shown to be hydrolyzed by both subtilisins: Arg₅-Ser₆, Ser₆-Ser₇, Arg₁₃-Pro₁₆, and Thr₂₀-Thr₂₁. A peak appearing early in the chromatogram contained only arginine and in large amounts. From the position in the elution pattern, (Arg)₂ or (Arg)₃ are the most likely forms of the liberated arginine.

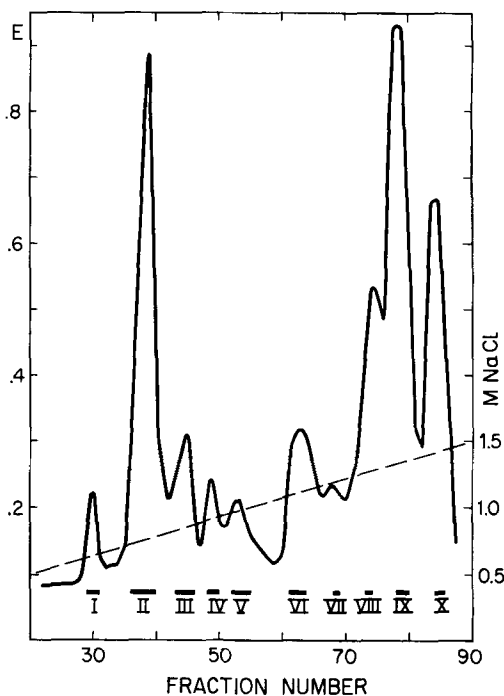
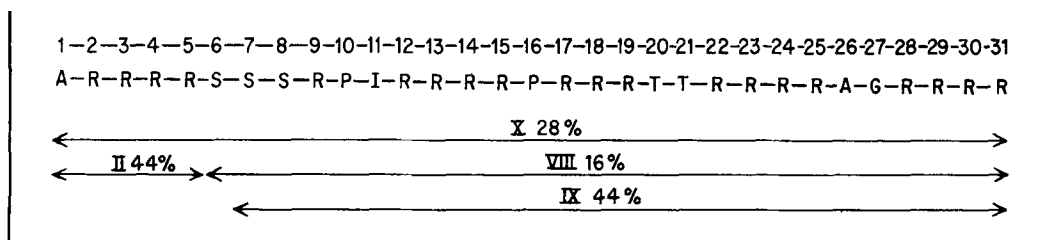


Figure 8. Chromatography of clupein Y^I, digested with nitrated subtilisin Carlsberg, on Whatman CM 52 (1 × 120 cm). Salt gradient from 0.5 to 1.5 M-NaCl. Flow-rate 15 ml/hr. 2.5 ml fractions collected. Room temperature. Abscisse: Fraction number. Left ordinate: E_{520 nm}. Right ordinate: NaCl conc (M). - - - salt gradient - fractions pooled for further analysis.

3.3.4 Nitrated subtilisin Carlsberg

When clupein-Z was cleaved by nitrated subtilisin Carlsberg, ion-exchange chromatography showed an elution pattern very similar to the one shown in Fig. 6. However, the dominating peak (VI in Fig. 6) was larger, peak VIII of equal size, and the rest smaller. Only peaks VI

Figure 9. Compositions and yields (in per cent of material applied to the Whatman CM 52 column) of peptide fragments from digest of clupein Y^I with nitrated subtilisin Carlsberg.



and VIII were analyzed further. The yields of the peptides are shown in Fig. 7, C.

Fig. 8 shows the elution pattern of peptides obtained by hydrolysis of clupein Y^I by nitrated subtilisin Carlsberg. In this case, the column length was 120 cm. The four peaks II, VIII, IX and X, which constitute the major portion of the eluted peptides, have all been identified. The peak number and per cent recovery of these peptides are shown in Fig. 9. Neither the peptide Ala₁-Ser₆ or a peak containing exclusively Arg, both of which were formed in the hydrolysis of Y^I by native subtilisin Carlsberg or Novo, have been identified as products of hydrolysis by the nitrated enzyme.

3.3.5 Potentiometric titrations

Potentiometric titration of clupein sulfate showed that the N-terminal amino groups titrate with an average pK of 7.6. Using this value and the amount of clupein in the hydrolyzed solution, the base consumption during the experiment (2 μ eq. in all experiments) indicate that 1.5 to 1.7 bond per molecule was hydrolyzed. This finding is in agreement with only two major cleavage-points being observed for clupein-Z and Y^{II}. Many more bonds are hydrolyzed if the reaction mixture is left for a long time. For Y^{II} chloride a total base-uptake of 10 μ eq. was recorded for a 0.5% solution, corresponding to the cleavage of 8 bonds.

4. DISCUSSION

Clupein, a mixture of three protamines isolated from herring sperms, is a good substrate for subtilisin Novo. It has been shown from studies with ester substrates or small synthetic peptides that the subtilisins preferentially split bonds adjacent to Tyr, Phe or Leu (8). None of these amino acid residues are present in clupein which furthermore contains about 65% of the positively charged arginyl residues. Therefore, it was of interest to determine which bonds were hydrolyzed in this substrate by the subtilisins in order to get a better understanding of the importance of the secondary binding sites in these enzymes.

From studies of the hydrolysis of synthetic peptides of different length and composition,

MORIHARA and coworkers (8) have shown that at least six residues of the substrate come into contact with the surface of the subtilisins upon binding. Four of these are located on the N-terminal side of the susceptible bond and two on the C-terminal side. This means, in the nomenclature of SCHECHTER and BERGER (14), that the subtilisins have at least six subsites (S₁ to S₄ and S'₁, S'₂). X-ray crystallographic studies by KRAUT and coworkers (13) have identified the part of the surface of subtilisin BPN' which constitutes subsites S₁ to S₄ for the synthetic peptides investigated. The preference for Tyr, Phe and Leu in S₁ was confirmed, and Ala was shown to be the ideal amino acid residue in S₂ since a larger side chain would get into too close contact with the enzyme. The side-chain accommodated in S₃ was not critical since it was pointing away from the enzyme. Finally, a hydrophobic side-chain is preferred by subsite S₄. The subsites S'₁ and S'₂ were not located by X-ray analysis, but are considered to be hydrophobic in nature («Sticky spots»).

Studies of the rate of hydrolysis of the individual clupein components (clupein Y^I, Y^{II} and Z) have shown that clupein Y^{II} and Z are hydrolyzed by subtilisin Novo with identical rates and faster than Y^I. Clupein Y^{II} and Z are nearly identical in sequences (see Fig. 10), and the bonds being hydrolyzed in the initial state of hydrolysis are identical in the two components. The first bond to be opened is Ser₂₁-Arg₂₂ in clupein Y^{II} (which corresponds to Ser₂₂-Arg₂₃ in clupein Z). This is followed by Ala₈-Ser₉ in clupein Y^{II} (Ala₉-Ser₁₀ in clupein Z). Many more bonds can be hydrolyzed in the two components, but with a rate which is slow compared to that with which the two bonds just mentioned are opened.

No obvious correspondance is obtained between the conclusions about subsites made from X-ray crystallographic studies of subtilisin BPN' and the sequence around the two bonds preferentially hydrolyzed in clupein Y^{II} and Z by this enzyme. In both cases the preferred hydrolysis of the bond Ser₂₁-Arg₂₂ (Ser₂₂-Arg₂₃, respectively) places a Ser in S₁, a bulky Val in S₂, a positively charged Arg in S₄ and two positively charged Arg's in S'₁ and S'₂.

Clupein Y^I is hydrolyzed with a slower rate than

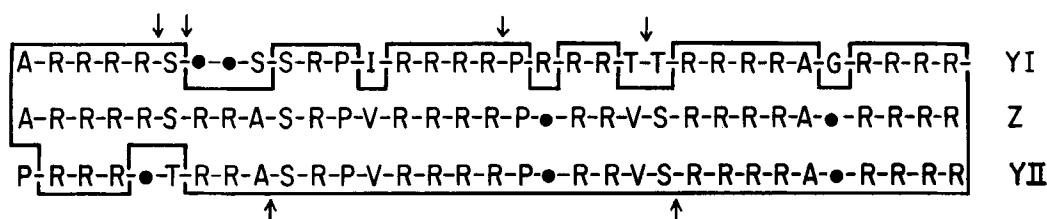


Figure 10. The amino acid sequence of clupein Y^I, Z and Y^{II} arranged to give maximal homology. ● indicates presumed deletions. † principal points of attack in clupein Y^{II} and Z. ‡: points of attack in clupein Y^I.

are the two other components. In addition, it appears that several bonds are hydrolyzed with about equal rates giving rise to a mixture of peptides not easily resolved on ion-exchange chromatography. Although the homology is close between Y^I and the two other components (see Fig. 10), one of the differences is seen at the susceptible Ser-Arg bond in Y^{II} and Z. The equivalent bond in Y^I is Thr-Arg. The close structural similarity between Ser and Thr argues against any major change in susceptibility to enzymatic attack. The replacement in subsite S₂ of a Val in Y^{II} or Z by a Thr in Y^I may be more important since a hydrophilic residue is replaced by a hydrophobic one. The remaining four subsites (S₃, S₄, S'₁ and S'₂) are occupied by identical residues (Arg) in all three substrates and should not contribute to the difference in rate of hydrolysis. This difference could perhaps also be due to strong non-productive binding of Y^I, but a detailed kinetic study of this possibility has not been attempted.

Subtilisin Carlsberg hydrolyzes the same two bonds in Y^{II} and Z as does subtilisin Novo although the rates with which they are opened are much slower. These results, therefore, confirm earlier studies (6) which indicated that the differences in the enzymatic activity between the two enzymes are quantitative rather than qualitative. Although a precise assignment of the peptides produced in the hydrolysis of Clupein Y^I has not been possible, the similarity in elution-patterns from ion-exchange chromatography suggest that also in this case the differences in enzymatic activity between the two enzymes are quantitative.

It has previously been shown that nitration of Tyr₁₀₄ in subtilisin Carlsberg results in a much faster rate of hydrolysis of clupein sulfate (17). When clupein-Z is hydrolyzed by nitrated subtilisin Carlsberg, the same bonds are split as with the unmodified enzyme. However, the relative rate with which the bonds Ser₂₂-Arg₂₃ and Ala₉-Ser₁₀ are opened has, to a marked degree, changed in favor of the former. KRAUT and coworkers have shown that Tyr₁₀₄ is part of subsite S₄ in subtilisin BPN' (identical to subtilisin Novo). A detailed X-ray crystallographic study of the Carlsberg enzyme has not yet been published but the close homology between the two subtilisins (20) strongly suggest that Tyr₁₀₄ is part of S₄ in subtilisin Carlsberg as well. (A model of subtilisin Carlsberg built in our laboratory from the coordinates for subtilisin BPN' is compatible with this suggestion). Since the same bonds are hydrolyzed by unmodified and modified subtilisin Carlsberg in clupein-Z, it is reasonable to suggest that it is an already existing binding mode, the one suggested by KRAUT and coworkers, which has been improved.

It has been suggested (18) that the negative charge which is introduced at pH 8 by nitration of Tyr₁₀₄ interacts with a positive arginyl side-chain and in this way facilitates the binding of clupein. When Ser₂₂-Arg₂₃ is hydrolyzed, an arginyl side-chain will bind in subsite S₄, and thus enhance the rate of hydrolysis. In contrast, a seryl side-chain will bind to S₄ when Ala₉-Ser₁₀ is hydrolyzed. Therefore, the hydrolysis of this bond should not be influenced by nitration of subtilisin Carlsberg, and the relative rates with which the two bonds are cleaved should change in favor of the Ser₂₂-Arg₂₃, which was found to be the case.

Hydrolysis of the bonds Arg₅-Ser₆, Ser₆-Ser₇, Arg₁₅-Pro₁₆ and Thr₂₀-Thr₂₁ which has been demonstrated in clupein Y^I also takes place

with an arginyl residues placed in S_4 . The enhancement in rate of hydrolysis which takes place with clupein- Y^I when subtilisin Carlsberg is nitrated thus can be explained, in part, in a similar way as for clupein-Z, since these bonds are also hydrolyzed by nitrated subtilisin Carlsberg.

As observed for clupein-Z hydrolysis, a change in preference of certain bonds takes place upon nitration. The hydrolysis of Arg_5-Ser_6 takes place only to a very limited extent by the unmodified subtilisin Carlsberg, but becomes the major point of attack by nitrated subtilisin Carlsberg (see Fig. 9). In addition, the large amounts of Arg_x liberated by the unmodified enzyme were not observed with the nitrated one.

The hydrolysis of all three clupein components involves bonds with serine as one of the donors (on either side). This may be more than a coincidence. Thus, when ovalbumin is transformed into plakalbumin, an Ala-Ser bond is broken (9, 19). When ribonuclease is transformed into ribonuclease-S, an Ala-Ser bond is split (12). It should be realized, naturally, that in these two cases the availability of these bonds are influenced by the folding of the proteins in the native state. Upon the complex formation between potato inhibitor II-b and subtilisin BPN', a Lys-Ser bond is cleaved in the inhibitor (4). Of the 10 bonds cleaved in glucagon by subtilisin Carlsberg (16), two involves a serine residue: Ser-Lys and Ser-Asp. When the B-chain of oxidized insulin is hydrolyzed by the subtilisins, a Ser-His bond is among the first bonds to be cleaved (6). Of the five bonds hydrolyzed in a peptide from lactic dehydrogenase, three involve serines: Ser-Gly, Asp-Ser and Ser-Ala (3). Finally, it should be mentioned that denatured subtilisin is an excellent substrate for native subtilisin. Since the enzymes contain many seryl residues (32 in the Carlsberg enzyme and 37 in subtilisin Novo), this may be the reason for the high rate of hydrolysis. Although many synthetic peptide- and ester substrates have been used in the study of the enzymatic activity of the subtilisins none of these have included a Ser. Thus, it is not possible to evaluate the importance of this side-chain relative to f.ex. Tyr.

The hydrolysis of the clupein components as their sulfate salts is only slightly effected by the presence of 0.1 M-NaCl. The corresponding chlorides, on the other hand, are hydrolyzed very slowly in distilled water, and addition of 0.1 M-NaCl was necessary to obtain rates of hydrolysis comparable to those of the sulfates. This difference probably reflects a difference in binding between the monovalent chloride-ion and the divalent sulfate-ion to the arginyl side-chains of clupein. Such a difference in ion-binding might lead to a difference in type of bonds being hydrolyzed by the subtilisins. To test this possibility, clupein-Z and Y^I chlorides were converted to the corresponding sulfates and hydrolyzed with subtilisin Novo. Analysis of the resulting peptides showed that the same bonds were hydrolyzed in the initial stage as when the chlorides were hydrolyzed. Thus, the counterion does not influence the mode of binding of clupein-Z and Y^I to subtilisin Novo. The same is probably true for the binding of clupein sulfate to subtilisin Carlsberg.

When the B-chain of oxidized insulin is cleaved by the subtilisins, these enzymes show a strong preference for a single bond, $Leu_{15}-Tyr_{16}$ (6). The hydrolysis of this bond is adequately explained by the excellent fit between the residues around the susceptible bond and the subsites identified by KRAUT and coworkers (13). When clupein Y^{II} or Z are hydrolyzed, a similar preference is also demonstrated which, in the case of subtilisin Carlsberg, can be enhanced by nitration (17). The main purpose of the present investigation was to determine why a substrate which does not contain amino acid residues that are good candidates for occupying subsite S_1 (Tyr, Leu, Phe), nonetheless is hydrolyzed rapidly by subtilisin Novo. It was surprising to find that the subtilisins have such strong preference for two bonds in clupein Y^{II} and Z and that the specificity towards the Ser-Arg bond appears to be dictated by the presence of a Val in P_2 . The hydrolysis of Ala-Ser rather than the adjacent Ser-Arg is presumably due to the inability of S_2 to accommodate a Pro (see Fig. 10). These findings add further evidence to the importance of secondary binding sites for both binding and specificity.

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