The Primary Structure of Calf Chymosin*

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Bent Foltmann, Vibeke Barkholt Pedersen, Dorothy Kaufman,‡ and Grith Wybrandt§

From the Institute of Biochemical Genetics, University of Copenhagen, DK-1353 Copenhagen K, Denmark

The complete amino acid sequence of calf chymosin (rennin) (EC 3.4.23.4) has been determined. The sequence consists of a single peptide chain of 323 amino acid residues. The primary structure of the precursor part of calf prochymosin was published previously (Pedersen, V. B., and Foltmann, B. (1975) Eur. J. Biochem. 55, 95–103). Thus we are now able to account for the total 365 amino acid residues of calf prochymosin.

Comparison of the sequence of calf prochymosin with that of pig pepsinogen A (EC 3.4.23.1) shows extensive homology. In the precursor part of the sequence, 15 residues are located at identical positions, as compared to 189 identical residues in the respective enzymes. Furthermore comparison to Penicillium janthinellum acid proteinase (penicillopepsin) (EC : 3.4.23.7) shows that 78 residues are common to this enzyme and to the two gastric proteinases. These homologies in sequence further suggest that the folding of the peptide chain in chymosin is very similar to that of other acid proteinases.

Chymosin (rennin) (EC 3.4.23.4) is the predominant milk-clotting acid proteinase in the fourth stomach of the calf, and the main proteolytic enzyme in calf cheese rennet. Like pepsin it is secreted as an inactive precursor which is irreversibly converted into active enzyme through limited proteolysis resulting in a final loss of the 42 NH₂-terminal residues of thezymogen (1).

The general biochemistry of prochymosin and chymosin was reviewed in 1966 (2). The enzyme may be regarded as a representative of one of the major types of gastric proteases. Furthermore x-ray crystallographers are working on the tertiary structure (3, 4) and consequently we considered it important to determine the primary structure of chymosin.

The first experiments were carried out with crystalline chymosin. These resulted in determinations of the sequence around the S—S bridges, at the NH₂ terminus and at the COOH terminus (5–7). The results indicated a high degree of homology between the primary structures of prochymosin and pepsin (8).

By chromatography on DEAE-cellulose the chymosin used in our experiments could be separated into two main components (chymosin A and B) (2). The final determination of the primary structure was performed with chromatographically purified preparations. In chymosin B all 323 residues were identified. Of 156 residues identified in chymosin A, only one was different in chymosin B. A preliminary report on the chymosin sequence has been published (9). This also includes the NH₂-terminal sequence of prochymosin (10).

The strategy we followed was conventional and started with the purification of fragments, either after cleavage by cyanogen bromide, or after tryptic digestion of amino-blocked enzyme. The large fragments prepared this way contained from 56 to 146 residues. After subsequent proteolytic degradations we were able to deduce an unambiguous structure, although in many experiments we did not recover all peptides. In the main text we describe an outline of the pertinent evidence. Details of materials and methods as well as results are given in the miniprint supplement.

While this work was in progress the amino acid sequence of porcine pepsinogen A was completed (11–14). Alignment of calf prochymosin against porcine pepsinogen A shows that 204 amino acid residues are common to the two zymogens.

**EXPERIMENTAL PROCEDURES**

**Fragmentation of the Peptide Chain**—Large fragments were obtained by cyanogen bromide cleavage (15, 16) or by tryptic digestion of protein in which amino groups were blocked by maleylation (17) or citraconylation (18). For such tryptic digestion we found a great improvement in specificity if the digestion was carried out at 12°C (10, 19). Although in some digestions we obtained only partial cleavage after arginine 203, well-defined tryptic fragments were obtained after digestion at 12°C.

Subdigestions were performed with the following enzymes: chymotryptsin, elastase, Staphylococcus aureus proteinase, thermolysin, and, after deblocking, trypsin or Armillaria mellea proteinase. The latter enzyme cleaves peptide bonds on the amino side of lysine residues (20). As described in the legend to Fig. 1, peptides are designated with letters indicating the digestions plus the number of the first and the last residue.

**Purification of Peptides**—Cyanogen bromide fragments were purified by gel filtration on Sephadex G-100 in 55% acetic acid. For purification of other aggregating fragments we used gel filtration on Sephadex G-100 in 0.05 M NH₄HCO₃ containing 8 M urea. The first step of fractionation of subdigests was in most cases gel filtration in volatile buffers such as 0.05 M NH₄HCO₃. The conditions of the individual separations are given in legends to the figures.

Final purification of low molecular weight peptides was generally obtained by high voltage paper electrophoresis or paper chromatography or both. Diagonal electrophoretic methods were preferentially used for purification of peptides containing cystine (21), methionine (22), or lysine (17).

**Sequence Determination**—All Edman degradations were performed manually. Carboxypeptidase A (23) and carboxypeptidase Y (24) were used for determinations of some COOH-terminal sequences.

1 In the sequence reported in Ref. 9, Fig. 2, misprints have occurred at positions 160 and 239.
2 Portions of this paper (including Figs. 4 to 23 and Tables 1 to 17) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-99, cite author(s), and include a check or money order for $8.10 per set of photocopies.
FIG. 1. The amino acid sequence of chymosin B with summary of the data from which the sequence was derived. The numbering starts from the NH$_2$ terminus of prochymosin. The peptide chain of chymosin starts at residue 45. Positions 208, 276, 338, 339, and 343 are gaps relative to the peptide chain of porcine pepsin. Results presented in this paper are marked by thick lines for residues whenever a series of digestions, with purification after each step has been essential for obtaining the peptide in question; e.g. A-C-S indicates digestion with A. mellea proteinase followed by digestion with chymotrypsin and finally with S. aureus proteinase. If the specificity of a preceding degradation determines the terminal amino acid residue of a given peptide, such degradations are also indicated. Disulfide bridges connect Cys 91 and Cys 96, Cys 252 and Cys 256, and Cys 296 and Cys 329.

RESULTS

The total sequence of chymosin B is presented in Fig. 1 together with a summary of evidence from which the sequence was derived. Previously published sequences (5, 6) that are important for the deduction are included in Fig. 1. To facilitate the comparison between the amino acid sequences of the gastric proteinases and of their zymogens we use a numbering of residues starting with the NH$_2$ terminus of the longest of the peptide chains (prochymosin) and count gaps where such occur in chymosin relative to pepsin. The numbering of amino acid residues in porcine pepsin A (13) may be converted to our zymogen numbering by adding 46. With the zymogen numbering the peptide chain of chymosin presented in this paper starts at residue 45.

The thermolytic peptides were obtained from a digest of intact chymosin. All other peptides were prepared after fragmentation with cyanogen bromide or digestion with trypsin. Cyanogen Bromide Fragments—Fig. 2 shows a flow diagram for purification of the cyanogen bromide fragments. After preliminary experiments with reduced and carboxy-
methylated or reduced and aminoethylated chymosin, it was found that fragments could be more easily purified if the cyanogen bromide cleavage was performed prior to reduction. After the first gel filtration (Fig. 5), CB 45-126 was obtained pure, and the low molecular weight peptides were isolated by paper electrophoresis. CB 211-302 and CB 314-373 were prepared after reduction, aminoethylation, and repeated gel filtrations (Figs. 6 and 7).

Tryptic Fragments—From the primary structure shown in Fig. 1 it can be seen that cleavage of S-S bridges does not alter the peptides produced from the tryptic digestion of maleylated or citraconylated chymosin. However, reduction of S-S bridges followed by modification of the -SH groups did influence the behavior of the fragments in gel filtration. Therefore, in most experiments chymosin was first reduced, aminoethylated, and then maleylated before digestion with trypsin. The purification of the tryptic fragments is summarized in Fig. 3, and the corresponding gel filtrations are listed. In these experiments, gel filtrations gave satisfactory separation of the three large TM fragments.

The low molecular weight tryptic peptides were purified by paper electrophoresis. However, TM 190-203 could not be purified by paper electrophoresis. It was eventually purified by elution of the application zone followed by gel filtration on Sephadex G-25.

Among the large TM fragments TM 204-354 has a size of a small protein. The subsequent cleavages were accomplished with a combination of degradation procedures: a) cleavage with cyanogen bromide and subsequent digestion with staphylococcal proteinase; b) deblocking and tryptic digestion; c) digestion with chymotryptic; d) deblocking and digestion with A. mellea proteinase followed by digestion either with chymotrypsin and then with staphylococcal proteinase, or digestion with elastase; e) digestion with staphylococcal proteinase.

From the sequences of the peptides obtained in the two major cleavages (Figs. 2 and 3), we are able to construct an unambiguous complete sequence of chymosin as shown in Fig. 1.

**DISCUSSION**

Comments on the experimental methods and possible genetic variants other than chymosin A and B are given in the
This discussion will be limited to a comparison between the structure of chymosin and those of other acid proteinases; of these, porcine pepsin and penicillopepsin (25) have been fully sequenced. Alignment shows that of 323 residues in chymosin, 189 are identical with the corresponding residues in porcine pepsin. Comparison between chymosin and penicillopepsin gives 89 common residues, porcine pepsin and penicillopepsin have 98 residues in common (25). Of the common residues, 76 are found at the same positions in all three enzymes. The homology in primary structures suggest a similar folding of the peptide chains of these enzymes. This assumption has been sustained by x-ray crystallography of the crystalline enzymes.

The tertiary structure of porcine pepsin has been determined by Andreeva et al. (26), and the structure of penicillopepsin has been determined by Hsu et al. (25). Two other acid proteinases from Rhizopus chinensis and from Endothia parasitica have also been analyzed by x-ray crystallography. Their primary structures are not yet completed, but the electron density maps show that they are closely related to each other (27) and to penicillopepsin (28). Furthermore data from x-ray crystallography of chymosin have been compared to the structure of E. parasitica proteinase. The results of the rotation function provide strong evidence for a homology between the two enzymes (4), and visual comparison between the tertiary structure of pepsin and the microbial proteinases also indicates homology between these enzymes.

From these investigations the following general structure appears: the molecules of the acid proteinases are bilobal with an extended hydrophobic interior. The two lobes are separated by a deep cleft. Behind the cleft there is a stack of mixed P-sheet, and the covalent connection between the two lobes is established by a strand running on the back of the P-sheet. Only a few turns of y-helices are found.

We are now able to see relationships between the tertiary structure, the highly homologous sections of the primary structures and the residues that are identified as catalytically active by inhibition studies. The enzymes are inhibited if one of two aspartic acid residues are esterified. Aspartic acid 78...
may be esterified with substrate-like epoxides (29, 30), while aspartic acid 261 reacts with diazo compounds (31, 32). Both of these groups are located in β bonds pointing toward the cleft of the molecule, and each bond is crossed by a strand which gives rise to ψ shaped structures (28). With the numbering used in Fig. 1 the residues of these structures are 77 to 81, crossed by 104 to 170, and 259 to 264, crossed by 347 to 352. Of the 24 residues in these four sections, 19 are identical in all three enzymes. This high degree of identity strongly supports the view that the catalytic apparatus is the same. James et al. (33) have suggested that tyrosine 121 also participates in the catalytic mechanism, and again we observe 6 identical residues from residue 118 to 125.

Other highly homologous sections are found in the regions that connect the two lobes. Extensive hydrophobic interactions are found in the antiparallel pleated sheet formed by residue 196 to 200 and 356 to 361. In these sections of the peptide chain there is only one substitution between pepsin and chymosin and 7 residues are common to all three enzymes. The covalent connection between the two lobes goes from glycine 214 to leucine 225; here 9 residues are common.

The eight sections mentioned here comprise 53 residues, of which 41 are common to all three enzymes. The rest of the identical residues are more evenly distributed, and the significance of these is not obvious with the present information.

With the present knowledge we are not able to comment on the high milk-clotting and low proteolytic activity of chymosin as compared to pepsin. Small differences in the binding sites of the cleft may be responsible. However, if we assume that the tertiary structure of chymosin corresponds to that of other acid proteases, we may have an explanation for the difference in milk-clotting activity between chymosin A and B (2). The only difference in structure that has been found so far is that residue 290 is aspartic acid in chymosin A and glycine in chymosin B. In the tertiary structure this residue is located in the opening of the substrate binding cleft; this will most likely influence the binding of substrate.

In addition to porcine pepsinogen and calf prochymosin, fragmentary information is available for another seven vertebrate pepsinogens and pepsins (34). Among these, the NH2-terminal sequence of bovine pepsinogen A is the longest terminal sequence of bovine pepsinogen A is the longest coherent sequence published. Out of 110 amino acid residues, while only 57 residues or 52% are common to bovine pepsin and chymosin B. In the tertiary structure this residue is located in the opening of the substrate binding cleft; this will most likely influence the binding of substrate.

At the moment it is premature to attempt a detailed discussion on the phylogenetic relationships among the acid proteases, but the results suggest that the chymosins represent a group of homologous enzymes that have evolved independently of the pepsins before divergence of the lines leading to cow and pig.

Acknowledgments—Carboxypeptidase Y, Staphylococcus aureus proteinase, and Armillaria mellea proteinase were gifts from Dr. H. Hayashi, Dr. D. Drapeau, and Dr. D. Smyth, respectively. The investigations on chymosin A were mainly carried out by Dr. H. Jacobsen. Mrs. Inge Hughe, and Mr. R. A. C. Coates have contributed with valuable technical assistance. We thank all for their collaboration.

REFERENCES


 Additional references are found on p. 8546.
Primary Structure of Chymosin

Adenyllytransferase was performed by the following method:

1. Addition of a solution containing the enzyme to a reaction mixture containing ATP, MgCl₂, and a substrate.

2. Incubation at a specific temperature and pH.

3. Measurement of the increase in absorbance at a specific wavelength.

RESULTS

Fig. 1. The activation of chymosin obtained after renneting

Fig. 3. The inhibition of renneting.

Fig. 5. The inhibition of renneting.

Table 1

<table>
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<th>Condition</th>
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<td>With Y</td>
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DISCUSSION

The results obtained in this study suggest that the addition of ATP and MgCl₂ to the reaction mixture can significantly enhance the activity of chymosin. The increase in absorbance at a specific wavelength indicates the formation of adenosine diphosphate (ADP), which is a key intermediate in the activation process.

Further studies are needed to investigate the mechanism of the activation process and to optimize the conditions for the highest activity of chymosin.
Primary Structure of Chymosin

The primary structure of chymosin has been determined by various methods, including mass spectrometry and X-ray crystallography. The complete amino acid sequence of chymosin from bovine stomach contains 393 amino acids, with a molecular weight of 42,000 daltons. Chymosin is an example of a proteolytic enzyme that is activated by calcium ions.

Table 1

<table>
<thead>
<tr>
<th>Amino Acid Composition of Aspartic Proteinase</th>
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<th>Asparagine</th>
<th>Glutamine</th>
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Table 2

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<th>Cysteine Oxidized Predicted</th>
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Table 3

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<th>Amino Acid Composition of Collagenase</th>
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Table 4

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<th>Glutamine</th>
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Fig. 2. The amino acid composition of chymosin.

Fig. 3. The primary structure of chymosin.

Fig. 4. The secondary structure of chymosin.

Fig. 5. The tertiary structure of chymosin.
Primary Structure of Chymosin

Table 1

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino Acid Composition</th>
<th>Peptides</th>
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Figure 1: Amino acid composition of peptides from 1970-1979.

Figure 2: Amino acid composition of peptides from 1980-1989.

Figure 3: Amino acid composition of peptides from 1990-1999.

Figure 4: Amino acid composition of peptides from 2000-2009.

Figure 5: Amino acid composition of peptides from 2010-2019.

Figure 6: Amino acid composition of peptides from 2020-2029.

Figure 7: Amino acid composition of peptides from 2030-2039.

Figure 8: Amino acid composition of peptides from 2040-2049.

Figure 9: Amino acid composition of peptides from 2050-2059.

Figure 10: Amino acid composition of peptides from 2060-2069.

Figure 11: Amino acid composition of peptides from 2070-2079.

Figure 12: Amino acid composition of peptides from 2080-2089.

Figure 13: Amino acid composition of peptides from 2090-2099.

Figure 14: Amino acid composition of peptides from 2100-2109.

Figure 15: Amino acid composition of peptides from 2110-2119.

Figure 16: Amino acid composition of peptides from 2120-2129.

Figure 17: Amino acid composition of peptides from 2130-2139.

Figure 18: Amino acid composition of peptides from 2140-2149.

Figure 19: Amino acid composition of peptides from 2150-2159.

Figure 20: Amino acid composition of peptides from 2160-2169.

Figure 21: Amino acid composition of peptides from 2170-2179.

Figure 22: Amino acid composition of peptides from 2180-2189.

Figure 23: Amino acid composition of peptides from 2190-2199.

Figure 24: Amino acid composition of peptides from 2200-2209.

Figure 25: Amino acid composition of peptides from 2210-2219.

Figure 26: Amino acid composition of peptides from 2220-2229.

Figure 27: Amino acid composition of peptides from 2230-2239.

Figure 28: Amino acid composition of peptides from 2240-2249.

Figure 29: Amino acid composition of peptides from 2250-2259.

Figure 30: Amino acid composition of peptides from 2260-2269.

Figure 31: Amino acid composition of peptides from 2270-2279.

Figure 32: Amino acid composition of peptides from 2280-2289.

Figure 33: Amino acid composition of peptides from 2290-2299.

Figure 34: Amino acid composition of peptides from 2300-2309.

Figure 35: Amino acid composition of peptides from 2310-2319.

Figure 36: Amino acid composition of peptides from 2320-2329.

Figure 37: Amino acid composition of peptides from 2330-2339.

Figure 38: Amino acid composition of peptides from 2340-2349.

Figure 39: Amino acid composition of peptides from 2350-2359.

Figure 40: Amino acid composition of peptides from 2360-2369.

Figure 41: Amino acid composition of peptides from 2370-2379.

Figure 42: Amino acid composition of peptides from 2380-2389.

Figure 43: Amino acid composition of peptides from 2390-2399.

Figure 44: Amino acid composition of peptides from 2400-2409.

Figure 45: Amino acid composition of peptides from 2410-2419.

Figure 46: Amino acid composition of peptides from 2420-2429.

Figure 47: Amino acid composition of peptides from 2430-2439.

Figure 48: Amino acid composition of peptides from 2440-2449.

Figure 49: Amino acid composition of peptides from 2450-2459.

Figure 50: Amino acid composition of peptides from 2460-2469.

Figure 51: Amino acid composition of peptides from 2470-2479.

Figure 52: Amino acid composition of peptides from 2480-2489.

Figure 53: Amino acid composition of peptides from 2490-2499.

Figure 54: Amino acid composition of peptides from 2500-2509.

Figure 55: Amino acid composition of peptides from 2510-2519.

Figure 56: Amino acid composition of peptides from 2520-2529.

Figure 57: Amino acid composition of peptides from 2530-2539.

Figure 58: Amino acid composition of peptides from 2540-2549.

Figure 59: Amino acid composition of peptides from 2550-2559.

Figure 60: Amino acid composition of peptides from 2560-2569.

Figure 61: Amino acid composition of peptides from 2570-2579.

Figure 62: Amino acid composition of peptides from 2580-2589.

Figure 63: Amino acid composition of peptides from 2590-2599.

Figure 64: Amino acid composition of peptides from 2600-2609.

Figure 65: Amino acid composition of peptides from 2610-2619.

Figure 66: Amino acid composition of peptides from 2620-2629.

Figure 67: Amino acid composition of peptides from 2630-2639.

Figure 68: Amino acid composition of peptides from 2640-2649.

Figure 69: Amino acid composition of peptides from 2650-2659.

Figure 70: Amino acid composition of peptides from 2660-2669.

Figure 71: Amino acid composition of peptides from 2670-2679.

Figure 72: Amino acid composition of peptides from 2680-2689.

Figure 73: Amino acid composition of peptides from 2690-2699.

Figure 74: Amino acid composition of peptides from 2700-2709.

Figure 75: Amino acid composition of peptides from 2710-2719.

Figure 76: Amino acid composition of peptides from 2720-2729.

Figure 77: Amino acid composition of peptides from 2730-2739.

Figure 78: Amino acid composition of peptides from 2740-2749.

Figure 79: Amino acid composition of peptides from 2750-2759.

Figure 80: Amino acid composition of peptides from 2760-2769.

Figure 81: Amino acid composition of peptides from 2770-2779.

Figure 82: Amino acid composition of peptides from 2780-2789.

Figure 83: Amino acid composition of peptides from 2790-2799.

Figure 84: Amino acid composition of peptides from 2800-2809.

Figure 85: Amino acid composition of peptides from 2810-2819.

Figure 86: Amino acid composition of peptides from 2820-2829.

Figure 87: Amino acid composition of peptides from 2830-2839.

Figure 88: Amino acid composition of peptides from 2840-2849.

Figure 89: Amino acid composition of peptides from 2850-2859.

Figure 90: Amino acid composition of peptides from 2860-2869.

Figure 91: Amino acid composition of peptides from 2870-2879.

Figure 92: Amino acid composition of peptides from 2880-2889.

Figure 93: Amino acid composition of peptides from 2890-2899.

Figure 94: Amino acid composition of peptides from 2900-2909.

Figure 95: Amino acid composition of peptides from 2910-2919.

Figure 96: Amino acid composition of peptides from 2920-2929.

Figure 97: Amino acid composition of peptides from 2930-2939.

Figure 98: Amino acid composition of peptides from 2940-2949.

Figure 99: Amino acid composition of peptides from 2950-2959.
Primary Structure of Chymosin

Table 1. Analysis of chymosin peptides.

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Table 2. Peptide mapping of chymosin.

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Figure 1. Chromatographic profiles of chymosin peptides.

Figure 2. Peptide mapping of chymosin.

Figure 3. Peptide mapping of chymosin.

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Primary Structure of Chymosin

Experiments of experimental methods

Table 1. Details of Chymosin preparation and some properties of the enzyme.

<table>
<thead>
<tr>
<th>Property</th>
<th>Details</th>
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<tbody>
<tr>
<td>Source of enzyme</td>
<td>Bakers' yeast</td>
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<tr>
<td>Purity</td>
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<tr>
<td>Specific activity</td>
<td>50 U/mg</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>60 kDa</td>
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</tbody>
</table>

The enzyme was purified from Bakers' yeast using ion-exchange chromatography and ultrafiltration. The specific activity of the purified enzyme was >95% and the molecular weight was 60 kDa. The purified enzyme was stored at -20°C and used for subsequent experiments.

References

44. van Kats, P.J., and Gelinas, J. (1973) J. Biol. Chem. 248, 245-252

The primary structure of calf chymosin.
B Foltmann, V B Pedersen, D Kauffman and G Wybrandt


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