Delineation of chicken cathepsin L secondary structure, relationship between pH dependence activity and helix content

E. Dufour, V. Dive, F. Toma

► To cite this version:

E. Dufour, V. Dive, F. Toma. Delineation of chicken cathepsin L secondary structure, relationship between pH dependence activity and helix content. BBA - Biochimica et Biophysica Acta, 1988, 955, pp.58-64. hal-02721019

HAL Id: hal-02721019

<https://hal.inrae.fr/hal-02721019>

Submitted on 1 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

BBA 33152

Delineation of chicken cathepsin L secondary structure; relationship between pH dependence activity and helix content

Eric Dufour ^a, Vincent Dive ^b and Flavio Toma ^b

^a INRA Theix, Ceyrat and ^b Service de Biochimie, CEN Saclay, Gif / Yvette (France)

(Received 13 January 1988)

Key words: Cathepsin L; Cysteine proteinase; Circular dichroism; Secondary structure; (Chicken)

The secondary structure of the recently sequenced chicken liver cathepsin L (EC 3.4.22.15) has been studied both by circular dichroism and a predictive method. The structural data provided by these approaches allow us to underline the extent of the structural similarities between cathepsin L and papain, one of the best known proteins in the cysteine proteinase family. The predictive method of Garnier et al. (*J. Mol. Biol.* 120 (1978) 97–120) is used to locate α -helix and β -sheet segments in the cathepsin L sequence. An optimization of decision constants has been performed, using circular dichroism data, to improve good predictions. The combination of these approaches lead us to suggest that the location of ordered structures observed in papain is maintained in cathepsin L, but with an additional α -helix in the middle region (residues 85–108) of cathepsin L. Furthermore, we show that cathepsin L inactivation at neutral pH is correlated to the lost of α -helix content (40% at pH 5.8 and 17% at pH 7.0) in this protein. It appears that such an effect can be related to the change in the ionization state of histidine side-chains which are shown to be mainly located in the predicted α -helix regions.

Introduction

The cysteine proteinase family includes mainly plant thiol proteinases – papain [1] and actinidin [2] – and animal lysosomal thiol proteinases – cathepsins B, H and L [3]. The lysosomal cysteine proteinases play an important role in intracellular protein catabolism [4] and possibly in the activation of peptide hormones [5]. Papain and actinidin, which are easily purified in large amounts, have been sequenced and their three-dimensional structures have been determined at high resolution

[6,7]. Comparison of their three-dimensional structure has shown that, despite 48% identity in their amino-acid sequence, the folding of the polypeptide chain in papain and actinidin is very similar [8]. The amino-acid sequences of cathepsins B and H have been determined by Takio et al. [9], whereas the primary structure of cathepsin L has been reported recently [10,11]. Comparison of the primary structures of plant cysteine proteinases (papain and actinidin) and animal lysosomal cysteine proteinases (cathepsins B, H and L) has shown that all these enzymes exhibited 35 to 50% sequence homology [8,11]. Such observations suggest that the tertiary structure of cathepsins B, H and L could be related to the tertiary structure of plant thiol proteinases.

In that respect, the secondary structure of cathepsin L has been presently approached by both circular dichroism and predictive method,

Abbreviations: CD, circular dichroism; Z, benzyloxycarbonyl; NMec, 4-methyl-7-coumarylamide.

Correspondence: E. Dufour, INRA de Theix, 63122 Ceyrat, France.

and has been compared to the secondary structure of papain as reference. From the CD spectra of cathepsin L at pH 5.8, as compared to that of papain in the same conditions, it appears that cathepsin L contains an higher α -helix content than papain. On the other hand, the method of Garnier et al. [12] suggests that the main ordered structures observed in papain are retained in cathepsin L; however, the algorithm predicts an additional α -helix in the middle region (residues 85–108) of the cathepsin L sequence. We also report that avian cathepsin L is rapidly inactivated at neutral pH and show by circular dichroism that this pH inactivation of the enzyme is related to secondary structural changes, i.e., the diminution of the α -helix content, as pointed out by the CD spectra. As, over the five histidine residues present in the cathepsin L sequence, four are located in α -helix segments, predicted by the method of Garnier et al. [12], we propose that the imidazolium deprotonation of histidines could be responsible for the helix breakage observed experimentally.

Materials and Methods

Materials

Papain was from Sigma (St Louis, MO). Chicken liver cathepsin L was purified as previously described [11]. Protein concentrations were determined using bovine serum albumin kit (Pierce, Rockford, IL) and ranged from 0.3 to 0.5 mg · ml⁻¹. Gel filtration was performed on a TSK 3000 SW column (Beckman, San Ramon, CA) using a 680 system controller, two 510 pumps and a 441 detector ($\lambda = 220$ nm) from Waters.

CD studies

For CD measurements, cathepsin L was dialysed for 4 h against 0.1 M phosphate buffer (pH 5.8) and papain was dissolved in the same buffer. CD studies (Jobin-Yvon Mark III dichrograph) were carried out at pH 5.8 and 7.0. The cylindrical cells used have a pathlength of 0.02 cm in the far ultraviolet and 1 cm in the near ultraviolet. The results are expressed in molar ellipticity [θ] (deg · cm² · dmole⁻¹) for the aromatic region and in mean residue weight (MRW) ellipticity for the peptide region. Experimental data were treated on

a Bull Micral 80-31 computer. The methods of Greenfield and Fasman [13] and Chen and Yang [14] were used to simulate the experimental spectra.

Enzyme assay

The assay for Z-Phe-Arg-NMec was carried out according to Barrett [15].

Prediction of secondary structure

The method of Garnier et al. (or GOR method [12]) was used; this algorithm predicted residues in four states: H, helix; E, sheet; turn is T and coil is C. The decision constantes were optimized to improve good predictions using CD results as previously reported [12].

Results

Instability of cathepsin L at neutral pH

As shown in Fig. 1, the activity of purified cathepsin L is optimum in a pH range of 5.0–6.0 and 90% of its activity is maintained after 40 min at pH 5.0. However, chicken liver cathepsin L is very unstable at neutral pH and 97% of its activity is lost after only 10 min incubation at pH 7.0. The pH inactivation of cathepsin L is not due to autolysis, because the enzyme was pre-incubated in absence of dithiothreitol and in this condition cysteine proteinases are unactivated. But the pro-

Fig. 1. Effect of the pH on the activity of chicken liver cathepsin L. Purified cathepsin L was pre-incubated without thiol activator, at 37 °C and at different pH for 2 min (---), 10 min (—) and 40 min (---). The buffers used were 0.1 M sodium acetate (pH 4.0–6.0) and 0.1 M sodium phosphate (pH 6.5–7.5). Activity was then measured using standard assay conditions and Z-Phe-Arg-NMec [15].

file of the curve suggests that histidine residues could be involved in the process (apparent $pK_a = 6.3$).

Circular dichroism spectra

The CD spectra of cathepsin L in 0.1 M phosphate at pH 5.8 and 7.0 in both the peptide region (190–260 nm) and the aromatic region (250–320 nm) are reported in Figs. 2A and 3A, respectively. Under this condition (0.1 M phosphate) it has been found by gel filtration on a TSK 3000 SW column that purified cathepsin L is in monomeric state (data not shown). Therefore, the spectra presented in Figs. 2A and 3A refer to this form of the enzyme in solution. Also shown as reference are the spectra of papain in the same conditions of buffer (Figs. 2B and 3B). While the two proteins display at pH 5.8 two minima near 210 and 220 nm, surprisingly cathepsin L, but not papain, exhibits a strong positive band at 192 nm (Fig. 2A). For papain, the CD spectra at pH 5.8 and 7.0 are

identical, whereas, at neutral pH, cathepsin L displays a very different spectrum: the large negative band around 215 nm was shifted to the low wavelength (200 nm) and the positive band disappears (Fig. 2A). Circular dichroism spectra can be seen as a linear combination of conformationally related subspectra. Hence, the fractions of α -helix, β -sheet and aperiodic segments (β -turn and random coil) for papain and cathepsin L could be estimated (Table I) from their CD spectra with those of models chosen as reference. In the present work, experimental CD spectra of cathepsin L and papain have been analysed by the method of Chen and Yang [14], who use the CD spectra of proteins of known secondary structure to derive the reference spectra. From this method the results presented in Table I show that cathepsin L exhibits an higher content of α -helix (40%) than papain (26%), whereas β -sheet contents are nearly identical at pH 5.8. At neutral pH, the α -helix content of cathepsin L decreases dramatically

Fig. 2. Far-ultraviolet CD spectra of cathepsin L (A) and papain (B) at pH 5.8 (—) and pH 7.0 (---). Calculated curves (.....) for cathepsin L and papain (method of Chen and Yang [14]).

Fig. 3. Near-ultraviolet CD spectra of cathepsin L (A) and papain (B) at pH 5.8 (—) and pH 7.0 (---).

(17%), whereas the random form reaches 60%, and the β -sheet content is kept constant. Papain exhibits the same contents of α -helix and β -sheet at the two pH values.

In the near-ultraviolet region, at pH 5.8, the two cysteine proteinases have qualitatively nearly

identical spectra showing the same substructures and located at the same wavelength: a negative band at 300 nm and positive shoulders at 265, 275 and 285 nm, and differing only in the intensities (Fig. 3). The CD spectra of papain are identical at pH 7.0 and 5.8, whereas cathepsin L displays

TABLE I
ESTIMATION OF α -HELIX AND β -SHEET CONTENT IN PAPAIN AND CHICKEN LIVER CATHEPSIN L

	X-ray data (6)	Chen and Yang method
Papain (pH 5.8 and 7.0)		
% α -helix	26	25
% β -sheet	17	15
Cathepsin L (pH 5.8)		
% α -helix	n.a. ^a	40
% β -sheet	n.a. ^a	21
Cathepsin L (pH 7.0)		
% α -helix	n.a. ^a	18
% β -sheet	n.a. ^a	21

^a n.a.: data not available.

TABLE II
PERCENTAGE OF α -HELIX AND β -SHEET PREDICTED BY THE METHOD OF GARNIER et al. [12] FOR PAPAIN AND CATHEPSIN L

	Papain			Cathepsin L		
	%H	%E	accuracy index (%)	%H	%E	
			H	E	total	
GOR method						
DC = 0	5	38	19	70	43 ^a	16 18
Optimized DC ^b	27	18	58	45	50 ^a	39 22

^a Good predictions over the four states, H, E, T and C.

^b The values of optimized decision constants (DC) are: papain, $DC_H = 120$ and $DC_E = -40$; cathepsin L, $DC_H = 140$ and $DC_E = 45$.

different spectra: the negative band (300 nm) and the positive band at 285 nm disappear, and the shoulders at 265 and 275 nm are shifted down.

Prediction of secondary structure

The predictive method of Garnier et al. [12] has been used to predict the location of α -helices and

	10	20	30	40	50
<u>Cathepsin L</u>	: APRSVDWREKGYVTPVKDQGCSCWAFNITGALEGQHFFKTGKLVSLSE				
DC = 0	: CCTCCTTCTTTTCCCTTTTCTTTTCTCCCCCHHHHHHHHTTCCCCCCC				
optimized DC	: CCC <u>HHHHH</u> TTTTTCEEEETTTTTTTEEECCCC <u>HHHHHHHHHH</u> T <u>HHHHHHH</u>				
<u>Papain</u>	: IPEYVDWRQKGAVTVPKNQSCGSCWAFSAVVTIEGIIKIRTGNLNQYSE				
DC = 0	: CEEETTCCTTTCCEETTTTTTTTTTCCCC <u>HHHHHH</u> EEEEETCCCCC <u>H</u>				
optimized DC	: CCT <u>HHHHHH</u> TCCCCCTTTTTTTTTT <u>HHHHHHHHHHHH</u> CCTTCCCCC <u>HH</u>				
X-ray data	: CCCCCTTTTTTCCCCCCCCCCCC <u>HHHHHHHHHHHHHHHHHH</u> CCCCC <u>H</u>				
	60	70	80	90	
<u>Cathepsin L</u>	: QNLVDCSRPEGNQGCNGGLMDQAFQYVQDNGGIDSEESYPYAKD.DC..				
DC = 0	: TTTCTCTCTTTTTTTTTTCCEEEEEEETTTCCCCCTTCTTCCCT.TH..				
optimized DC	: <u>HHHEEE</u> CCTTTTTTTTTTCCCCCEEEETTTCCCCC <u>HHHHHHHH</u> . <u>HH</u> ..				
<u>Papain</u>	: QELDCDR..RSYGCNGGYPWSALQLV.AQYGIHYRNTYPYEGVQRYCRS				
DC = 0	: <u>HHHH</u> TTTT..TTTTTTTTCTEEEEEE..EEEEETTCCTTTTEEEETTT				
optimized DC	: <u>HHHHHH</u> HT..TTTTTTCTCT <u>HHHHHH</u> . <u>HH</u> EEEEETTCCTTTTCCCCCTTT				
X-ray data	: <u>HHHHHH</u> TT..TTTTTCT <u>HHHHHHHHHH</u> . <u>HH</u> CCCTTTTCCCCCCCCCTT				
	100	110	120	130	140
<u>Cathepsin L</u>	: RYKAEYNAANDTGFVDIPQGERALMKAVASVGPVSAIDAGHSSFQFYQ				
DC = 0	: <u>HHHHHHHHHH</u> TTTEEECC <u>HHHHHHHHHH</u> HEEECCEEEECCCCCCCTCTT				
optimized DC	: <u>HHHHHHHHHHHH</u> HEEECHHHHHHHHHHHHHHHHEEEEECCCCCCEEEET				
<u>Papain</u>	: REKGPY.AAKTDGVRQVQPYNOGALLYSIA.NQPVSVVLQAAGKDFQLYR				
DC = 0	: TTTCTT.CCCTTEEEEEECTTTEEEEEEE.CCCEEEECCTCEEEET				
optimized DC	: TTTCC <u>H</u> . <u>HHHH</u> TTTEEEECTTTEEEEEEC.CCCEEE <u>HHHHHH</u> CCCTT				
X-ray data	: TTTCCC.CCCCCEEECCCC <u>HHHHHHHH</u> . <u>HC</u> CEEEEECC <u>HHHHHH</u> TT				
	150	160	170	180	
<u>Cathepsin L</u>	: SGIYEPDCSSEDLDHGVLVVGYGFEGGKKYWIWKNWSGKEKWDKGY..I				
DC = 0	: TTTTCTTTT <u>HHHHHH</u> EEEEEEECCTTTTTTEEECCCTCTTCTTTT..E				
optimized DC	: TTTEETTT <u>HHHHHHHH</u> EEEEEEECCTTCCCEEECCCCCTTCT <u>HHH</u> .. <u>H</u>				
<u>Papain</u>	: GGIFVGP.C.GNKVDHAVAAGVY...NPGYILIKNSWGTGWGENGYIRI				
DC = 0	: TTEEEET.T.TTCEEEEEEEETC...CTTEEEETTTCCCCCCTTEEE				
optimized DC	: TTEEEET.T.T <u>HHHHHHHH</u> HTC...CTTEEEECTCCCCCCTTEEE				
X-ray data	: CCEEECC.C.CCCEEEEEEE...ETTEEEEECCCCCCCCCEEE				
	190	200	210		
<u>Cathepsin L</u>	: .YMAKDRKNHCGIATAASYPLV				
DC = 0	: .EECCTTTTTTEEEEEECCTC				
optimized DC	: . <u>HHHHHHHHHH</u> EEEEECCTC				
<u>Papain</u>	: KRGTGNSYGVCGLYTSSFYPVKN				
DC = 0	: EETTTTTEEEEEETTTCCCTTC				
optimized DC	: ETTTTTTEEEEEETTTCCCTC				
X-ray data	: EECCTTTTTTTTTTCEEEEC				

Fig. 4. Prediction of cathepsin L secondary structure using the algorithm of Garnier et al. [12], with decision constants (DC) maintained equal to zero and optimized. Comparison with the papain secondary structure assignments from X-ray data and the α -helices and β -sheets predicted by the GOR method (DC = 0 and optimized DC) for papain. The residue numbers are those of papain.

β -sheets. The percentage and the location of α -helices and β -sheets for papain has first been assessed taking the decision constants equal to zero. In reference to X-ray data, the method gives a low percentage (Table II) of α -helix (5% instead of 27% for X-ray data). In the same way the GOR method predicts, for cathepsin L, 14% of α -helix instead of 40% determined by CD measurements. Considering the disagreement observed between the predictive method and the experimental data, an optimization of the decision constants has been performed for papain and cathepsin L, as suggested by Garnier et al. [12], using the α -helix and β -sheet contents deduced from X-ray and CD measurements, respectively. Applied to papain, this approach rises the accuracy of the prediction for α -helix from 19 to 58% (Table II). When the decision constants are maintained equal to zero, only two short α -helices (residues 31–36 and 50–54) are predicted by the method for papain, whereas with optimized decision constants, over the five α -helices determined by X-ray investigations, four are predicted by the GOR method (Fig. 4). In the case of cathepsin L, the optimized method of Garnier et al. predicts the location of seven α -helix segments (Fig. 4), which agrees with the 40% of α -helix structure deduced from the CD data. The main α -helix and β -sheet segments of papain are retained in cathepsin L (Fig. 4), especially the active-site helix (residues 25–42 in papain). However, a large α -helix (residues 85–108) is predicted for cathepsin L, but not papain, in the middle region. Papain and cathepsin L display similar β -sheet contents (Table I). For cathepsin L, the predicted β -sheet segments have to a great extent the same location as papain; however, the method of Garnier et al. predicts in the C-terminal part of cathepsin L sequence an α -helix, whereas papain exhibits a β -sheet segment (Fig. 4).

Discussion

Circular dichroism is a powerful method for the investigation of secondary structures and conformational changes in protein under various experimental conditions. Although several assumptions are inherent to the use of CD spectra to describe the secondary structure of proteins, as for example

the specific contributions of aromatic side-chains in the far-ultraviolet CD spectra, it has been shown for some proteins that this method gives reasonable accuracy in the secondary structure predictions of these proteins as compared to X-ray data [16]. Particularly, as long as aromatic side-chains do not account for more than 10% of the total residues in the protein, the method has been found to work for determining the α -helix content of a protein [17]. In fact, for papain the α -helix but also β -sheet contents deduced from the CD spectra are in excellent agreement with the radio-crystallographic data of this protein [6]. From this result, one could expect reliable results in the estimation of the secondary structure of cathepsin L, using the procedure of Chen and Yang [14]. One of the most striking features of the present work is the very different CD spectra displayed by cathepsin L in phosphate 0.1 M buffer (pH 5.8), as compared to that of papain under the same conditions. As these two proteins already contain the same amount of aromatic residues in their sequences (approx. 10%), and also show closely related near-ultraviolet CD spectra, we do not believe that aromatic contributions can account for the difference observed in the far-ultraviolet CD spectra of these proteins. This difference in CD spectra is therefore taken as strong evidence for the presence of a higher content of helical structure in cathepsin L.

Papain and actinidin are classified in the category of $\alpha + \beta$ proteins [18], in that α -helix and β -sheet regions are segregated along the polypeptide chain: one domain largely helical, while the other is built around an irregular twisted β -sheet. The tertiary structures of plant cysteine proteinases, despite 48% identity in their amino-acid sequence, are closely related [8]. Although no three-dimensional structural information is available for cathepsins, our results suggest that cathepsin L and papain, which display 45% homologies, have similar secondary structural elements. Nevertheless, cathepsin L and papain differ at least in two points: (i) CD studies show that cathepsin L has an higher α -helix content than papain, and (ii) cathepsin L is inactivated at neutral pH, but papain is not. The optimized method of Garnier et al. [12] predicts seven α -helix segments in the cathepsin L sequence: three are homologous with

papain (helices 25–42, 50–56 and 114–127), but two helices (155–160 and 191–200) are predicted in the C-terminal part of the cathepsin L sequence, a place where papain does not exhibit a helical segment. Nevertheless, one should remark that for papain the GOR method predicts an α -helix in position 156–164 instead of a β -sheet. As a consequence, the GOR method does not predict for cathepsin L two α -helix segments which are observed in the papain sequence (helices 66–79 and 138–143). However, it is interesting to notice that the method of Garnier et al. predicts an α -helix (residues 85–108) in the middle region of the cathepsin L sequence. This region (residues 82–116) in cysteine proteinase sequences, which lies exposed on the protein surface, is the less conserved [8]. In papain and actinidin sequences, most substitutions responsible for the largest difference in the three-dimensional structures are found in this region [8]. From these considerations, it seems that a simple explanation of the present experimental data (40% of helical structure for cathepsin L) should be a model in which the location of the α -helix segments are to a great extent the same as those observed in papain, but with an additional α -helix segment (residues 85–108) in the middle region of cathepsin L.

The plant cysteine proteinases have a broad pH optimum (5.0–7.0), whereas the cathepsins B, H and L are inactivated at neutral pH. Chicken cathepsin L is particularly unstable and the profile of the curve of inactivation suggests that histidine residues could be involved. One should also remark that if we except active site histidine (residue 159 in Fig. 4), found in all cysteine proteinases and which has an atypical pK_a of 4.2 (19), the cysteine proteinases with a broad pH range of activity contain no (actinidin) or one (papain) histidine residue in their sequence, whereas chicken cathepsin L exhibits four additional residues in its sequence. Furthermore, the CD data show that an increase in the pH from 5.8 to 7.0 produces a strong fall of the α -helix content in cathepsin L (40% at pH 5.8 and 17% at pH 7.0). Making the assumption that this pH modification promotes essentially a deprotonation of the histidine side-

chains, we have focused our attention on the location of the histidines in the cathepsin L sequence. Surprisingly, nearly all the histidine residues are found in the α -helix regions predicted by the method of Garnier et al. We may suggest therefore, that the destabilization of the helical structures observed at pH 7.0 results mainly in a change of the ionization state of one or more histidine residues located in α -helix segments. This loss of α -helix would explain the inactivation observed for chicken cathepsin L at pH 7.0.

References

- Mitchell, R.E.J., Chaiken, I.M. and Smith, E.L. (1970) *J. Biol. Chem.* 245, 3485–3492.
- McDowall, M.A. (1970) *Eur. J. Biochem.* 14, 214–221.
- Kirschke, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S. and Bohley, P. (1980) In *Protein Degradation in Health and Disease*, pp. 15–35, *Excerpta Medica*, Amsterdam.
- Barrett, A.J. and Kirschke, H. (1981) *Methods Enzymol.* 80, 535–561.
- Docherty, K., Carrell, R.J. and Steina, F. (1982) *Proc. Natl. Acad. Sci. USA* 79, 4613–4617.
- Drenth, J., Jansonius, J.N., Koekoek, J.N. and Wolthers, B.G. (1971) *Adv. Protein Chem.* 25, 79–115.
- Baker, E.N. (1980) *J. Mol. Biol.* 141, 441–484.
- Kamphuis, I.G., Drenth, J. and Baker, E.N. (1985) *J. Mol. Biol.* 182, 317–329.
- Takio, K., Tōwatari, K., Katunuma, N., Teller, D.C. and Titani, K. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3666–3670.
- Wada, K., Takai, T. and Tanabe, T. (1987) *Eur. J. Biochem.* 167, 13–18.
- Dufour, E., Obled, A., Valin, C., Bechet, D., Ribadeau-Dumas, B. and Huet, J.C. (1987) *Biochemistry* 26, 5689–5695.
- Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) *J. Mol. Biol.* 120, 97–120.
- Greenfield, N. and Fasman, G.D. (1969) *Biochemistry* 8, 4108–4116.
- Chen, Y.H. and Yang, J.T. (1971) *Biochem. Biophys. Res. Commun.* 44, 1285–1291.
- Barrett, A.J. (1980) *Biochem. J.* 187, 909–911.
- Woody, R.W. (1985) In *Peptides: analysis, synthesis and biology* (Undenfriend, S. and Meienhofer, J., eds.), pp. 16–104, Academic Press, London.
- Rosenkranz, H. (1974) *Z. Klin. Chem. Klin. Biochem.* 12, 415–422.
- Levitt, M. and Chothia, C. (1976) *Nature* 261, 552–558.
- Brocklehurst, K., Baines, B.S. and Kiersten, M.P.J. (1981) *Top. Enzyme Ferment. Biotechnol.* 5, 262–335.