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The Specificity of a Neutral Deoxyribonuclease from *Cancer pagurus*

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The recently isolated neutral deoxyribonuclease from crab (*Cancer pagurus*) testes has been characterized in its mode of action and its specificity. The enzyme is a typical endonuclease, forming 5′-phosphate oligonucleotides of large average size; after extensive digestion of calf thymus DNA over 75% of the fragments have a size larger than pentanucleotides and mononucleotides are absent. As far as specificity is concerned, thymidine is very abundant in the 5′-penultimate position (≈ 50%) and in the 3′-terminal position (37%) and almost absent in the 5′-terminal position (≈ 1%), the values quoted concerning *Escherichia coli* digests of average size ($\bar{P}_n$) between 50 and 10.

Recent results on the specificity of deoxyribonucleases (DNAases) and on their use in assessing the frequency of the short nucleotide sequences recognized and split by these enzymes (see [1] for a brief review) encouraged us to screen a number of deoxyribonucleases for specificity. In the present work, we have investigated the specificity of a neutral DNAase recently isolated from *Cancer pagurus* testes [2]. A new procedure of preparation of the enzyme, based on the method originally developed for spleen acid DNAase [3], has been set up. The specificity of the enzyme has been determined using the labelling and separation approaches recently established for the 3′-terminal nucleotides and the 5′-terminal dinucleotides [4,5] of the oligonucleotides formed by the crab DNAase. The 5′-terminal dinucleotides released from *Escherichia coli* DNA (51% dG + dC) by the enzyme are very characteristic in that thymidine is almost absent from the terminal position (≈ 1%) and predominates on the penultimate position (≈ 50%); thymidine also is the most frequent (37%) 3′-terminal nucleotide liberated by the enzyme.

Abbreviations. $\bar{P}_n$, average degree of polymerization, average size or average chain length of oligonucleotides. Abbreviations for nucleotides follow CBN Recommendations, see *Eur. J. Biochem.* *15*, 203–208 (1970).

Definitions. A_{280} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 280 nm, when measured in a 1-cm path-length cell. Isostichs are oligonucleotide size groups, regardless of their composition.

Enzymes. Acid phosphomonoesterase from porcine spleen (EC 3.1.3.2); neutral DNAase from crab testes (EC 3.1.4.).

MATERIALS AND METHODS

The DNA preparations used in the present work were described elsewhere [6,7].

Crab DNAase digestions were carried out in 0.005 M Tris-HCl pH 7.2, 0.01 M $MgCl_2$. DNA concentration and incubation temperature are indicated in the figure legends. Activity units are defined elsewhere [3].

The enzyme was inactivated by shaking the incubation mixture with 0.1 volume of chloroform–isoamyl alcohol (24:1; v/v) on a Whirlimixer (Springfield, Mass.). Digests were extensively dialyzed against distilled water, except where otherwise stated. For the determination of 5′-terminal dinucleotides, they were made 0.05 M in ammonium acetate buffer pH 4.6, and dephosphorylated by incubation at 37 °C for 4 h with 0.005 units of acid phosphatase B per A_{260} unit of digest; acid phosphatase was inactivated by shaking with chloroform–isoamyl alcohol, as indicated above.

DEAE-cellulose–urea column chromatography was done as previously described [6], on undialyzed digests showing a hyperchromic shift of 34%.

The methods for the determination of the 3′ and 5′-terminal nucleotides and the 5′-terminal dinucleotides have been described elsewhere [4,8,5] along with the enzyme preparations used.

The statistical evaluation of data was done using an Olivetti (Ivrea, Italy) Programma 102 desk computer (program 1A).

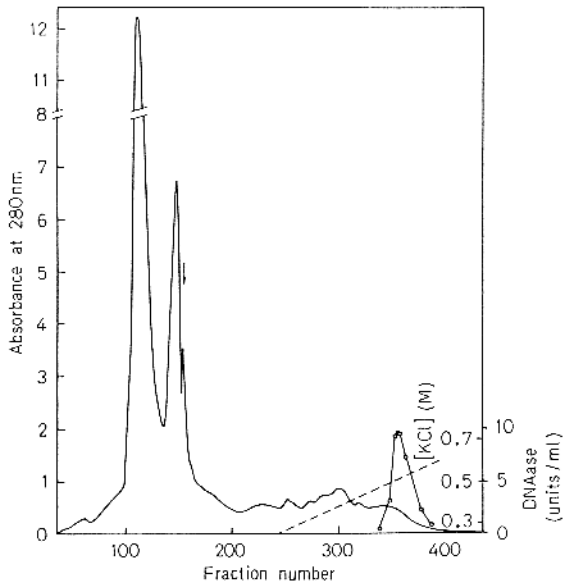

Fig. 1. *Chromatography of a crude crab-testes extract on DEAE-Sephadex A-50.* 420 ml of crude extract in 0.05 M phosphate buffer pH 6.8 (A_{280} = 21; A_{260} = 13.5) were loaded on a column (6 × 78 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was washed with the same buffer + 0.25 M KCl; a molarity gradient (0.25–0.75 M) of KCl in 0.05 M phosphate buffer was started where indicated by the arrow. 20-ml fractions were collected; the continuous line indicates the absorbance at 280 nm, circles DNase activity. The broken line indicates the KCl molarity. Fractions 352–375 were pooled, diluted with an equal volume of water and processed further

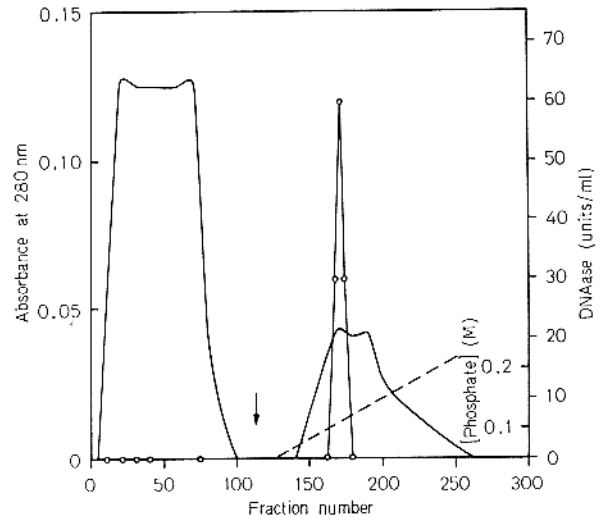

Fig. 2. *Chromatography of fractions 352–375 from the chromatogram of Fig. 1 on hydroxyapatite.* 960 ml (A_{280} = 0.175; A_{260} = 0.1) were loaded on a hydroxyapatite column (2 × 40 cm) equilibrated with 0.025 M phosphate buffer pH 6.8, 0.25 M KCl. The column was washed with the same buffer up to fraction 100 and then with 0.025 M phosphate buffer pH 6.8. At the fraction indicated by the arrow, a linear gradient (0.025–0.3 M) of phosphate buffer was started. 10-ml fraction were collected. The continuous line indicates the absorbance at 280 nm, circles indicate DNase activity. The broken line indicates the phosphate molarity. Fractions 168–172 were pooled and frozen

Table 1. *Chromatographic purification of crab neutral DNAase*

Fraction	Volume	Total	Activity/volume	Protein	Specific activity
	ml	units	units/ml	A_{280} units	units × ml^{-1} × A_{280}^{-1}
I. 0.15 M NaCl extract	5190	38925	7.5	65	0.1
II. Ammonium sulfate precipitate (80% satd)	990	12870	13	21	0.6
III. DEAE-Sephadex A-50[a]	490	3675	7.5	0.34	22
IV. Hydroxyapatite	45	2475	55	0.04	1375

[a] 420 ml (out of a total of 990 ml) were loaded.

RESULTS

Preparation of Crab DNAase

800 g of frozen crab tissues (testis and *vas deferens*) were homogenized in a Waring blendor in 3200 ml of 0.15 M NaCl and stirred for 1.5 h at room temperature. After 1 h of centrifugation at 9000 rev./min, supernatants were stored at 4 °C; the sediment was homogenized, stirred and centrifuged as just described.

Pooled supernatants (5190 ml) were fractionated between 40% and 80% saturation of ammonium sulfate. The precipitate at 80% saturation was dissolved in a small volume of distilled water and dialyzed against 0.05 M potassium phosphate, pH 6.8. This crude enzyme solution was split into two parts which were purified separately.

Two chromatographic steps were used to purify the enzyme, involving DEAE-Sephadex A-50 and hydroxyapatite columns, respectively (Fig. 1 and 2).

Table 1 summarizes the preparation and purification steps used.

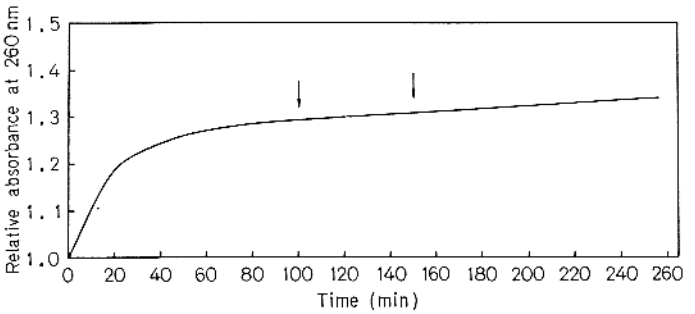

Fig. 3. *Hyperchromic shift of calf thymus DNA during* Cancer pagurus *DNAase digestion.* DNA (A_{260} = 8) in 0.005 M Tris-HCl pH 7.2, 0.01 M $MgCl_2$ was digested with 2.5 DNAase units. At the point indicated by the arrow the same amount of enzyme was added

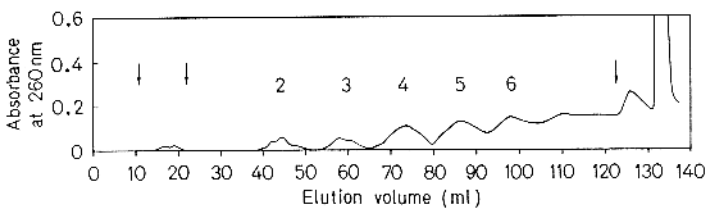

Fig. 4. *DEAE-cellulose—7-M-urea chromatography of* Cancer pagurus *DNAase digest.* 2 ml of calf thymus DNA was digested in the conditions described in the preceding figure to a hyperchromic shift of 34%. After inactivation of the enzyme, the digest was diluted twice with water and loaded on a DEAE-cellulose column (0.4 × 14 cm) equilibrated with 0.025 M ammonium acetate pH 5.5. The column was washed with 9 ml of water and 9 ml of 0.025 M ammonium acetate in 7 M urea. Oligonucleotides were eluted by a linear gradient of ammonium acetate (0.025—0.5 M) in 7 M urea. 1-ml fractions were collected. The arrows correspond to the water wash, the start of the ammonium acetate gradient and to a step of 0.5 M ammonium acetate in 7 M urea, respectively; the latter was followed by a step of 1 M ammonium acetate in 7 M urea at an elution volume equal to 140 ml

Kinetics of Calf-Thymus DNA Degradation and Isostich Distribution of the Digest

The distribution of the isostichs in the crab DNAase digest was investigated on digests having a hyperchromic shift of 34% (Fig. 3). Such a distribution is shown in Fig. 4 and is characterized by three main features: (a) in spite of the fact that digestion was pushed very far, over 75% of the fragments appeared to have a size larger than pentanucleotides and was not resolved by the column; (b) mononucleotides were absent from the digests as shown by experiments in which digests were run on DEAE-cellulose columns in the absence of urea; the first peak corresponding to the material eluted at the beginning of the chromatogram of Fig. 4 was digested with spleen phosphatase and with spleen exonuclease plus spleen phosphatase; neither digest showed nucleoside peaks when run on Aminex A6 columns [7], but only ultraviolet-absorbing material, mostly not retained by the resin; the peak labelled as 2 was shown to correspond to dinucleotides by a co-chromatography experiment with 5′-terminal ^{32}P-labelled dinucleotides; (c) a partial resolution of isostich components was observed in the dinucleotide and trinucleotide peak; tetranucleotides and pentanucleotides did not show symmetrical peak profiles.

Termini Released by the Crab DNAase

Three different DNAs, from calf thymus, *E. coli* and *Micrococcus luteus*, were digested to hyperchromic shifts equal to 10, 20 and 30% (which correspond to average sizes, $\bar{P}_n$, ranging from 50 to 10) [7]. 3′-Terminal nucleotides and 5′-terminal dinucleotides were determined on all hydrolysates. No significant differences were seen on hydrolysates obtained at different stages of degradation. Tables 2 and 3 show the data obtained. 5′-Terminal doublets are also plotted in the form of histograms in Fig. 5—7.

A direct determination of 5′-terminals as released from calf thymus DNA is also shown in Table 2. These results show very satisfactory agreement with data calculated from the 5′-terminal dinucleotides. In addition they show that the values for the 5′-dinucleotides starting with thymidine are overestimated.

DISCUSSION

Preparation Procedure

The original purification procedure of the crab enzyme [2] was not used in the present work since the enzyme yield of the original procedure was rather low (≈ 1%). The new purification procedure involves a combination of chromatographic steps which has proven very useful in the isolation of a number of enzymes in this laboratory. Basically, the two chromatographic steps used are patterned on the method developed for the purification of spleen acid DNAase [3]. Since the enzyme so obtained was free of contaminating activities interfering with the determination of specificity, no attempt was made to push the purification any further. It should be noticed that the increase in specific activity during purification is very high; this suggests that estimates in the early purification steps may have been affected by the presence of inhibitors.

Kinetics of DNA Degradation and Isostich Distribution

The isostich distribution of extensively degraded calf thymus DNA is characterized by the absence of

Table 2. *5'-Terminal dinucleotides released by crab DNAase*
5'-Terminal dinucleotides are written with the 5'-terminal mononucleotide on the left

5'-Terminal dinucleotides	*E. coli*	*M. luteus*	Calf thymus
	%		
dA-dA	3.1 ± 0.09	0.6 ± 0.10	3.1 ± 0.17
dA-dC	2.8 ± 0.13	2.8 ± 0.12	3.3 ± 0.13
dA-dG	3.9 ± 0.45	2.6 ± 0.40	5.0 ± 0.15
dA-dT	14.2 ± 0.35	3.5 ± 0.22	15.0 ± 0.75
dC-dA	6.6 ± 0.46	5.6 ± 0.27	8.4 ± 0.45
dC-dC	5.9 ± 0.67	9.6 ± 0.31	6.1 ± 0.27
dC-dG	7.4 ± 0.72	18.2 ± 1.21	0.8 ± 0.16
dC-dT	16.7 ± 0.73	15.3 ± 1.03	24.2 ± 1.53
dG-dA	4.8 ± 0.72	4.9 ± 0.69	6.8 ± 1.33
dG-dC	6.0 ± 0.33	9.2 ± 1.26	3.1 ± 0.22
dG-dG	7.4 ± 0.55	13.7 ± 0.75	6.7 ± 0.70
dG-dT	17.4 ± 0.81	12.8 ± 0.60	15.0 ± 0.61
dT-dA	0.5 ± 0.06	0.1 ± 0.05	0.4 ± 0.06
dT-dC	0.7 ± 0.10	0.4 ± 0.09	0.8 ± 0.29
dT-dG	1.0 ± 0.10	0.3 ± 0.04	0.5 ± 0.06
dT-dT	1.1 ± 0.32	0.2 ± 0.08	0.8 ± 0.25
5'-Terminal nucleotides			
dA	24.0 ± 0.68	9.1 ± 0.24	26.4 ± 0.94[a]
dC	36.6 ± 1.30	49.4 ± 2.37	39.4 ± 2.10[a]
dG	35.7 ± 1.69	40.3 ± 2.83	31.3 ± 1.99[a]
dT	3.3 ± 0.34	0.8 ± 0.13	2.7 ± 0.40[a]
5'-Penultimate nucleotides			
dA	15.0 ± 0.66	11.2 ± 0.68	18.5 ± 1.24
dC	15.5 ± 0.41	21.9 ± 1.29	13.3 ± 0.25
dG	19.6 ± 1.06	35.6 ± 1.18	12.9 ± 0.73
dT	49.4 ± 1.06	31.0 ± 1.21	55.0 ± 1.54
Number of determinations	7	6	9

[a] 5'-Terminals released from calf thymus DNA were also determined directly. The average of 8 determinations, at four different $\bar{P}_n$ values, were: dA 26.7 ± 0.53; dC 39.2 ± 0.68; dG 32.9 ± 0.36; dT 1.3 ± 0.20.

Table 3. *3'-Terminal nucleotides released by crab DNAase*

Nucleotide	Calf thymus	*E. coli*	*M. luteus*
	%		
dA	24.5 ± 0.59	22.8 ± 0.98	16.0 ± 1.25
dC	20.6 ± 0.50	24.1 ± 0.94	38.2 ± 0.47
dG	13.2 ± 0.66	16.1 ± 0.72	17.2 ± 0.47
dT	41.3 ± 1.03	36.9 ± 0.88	28.6 ± 0.61
Number of determinations	9	8	7

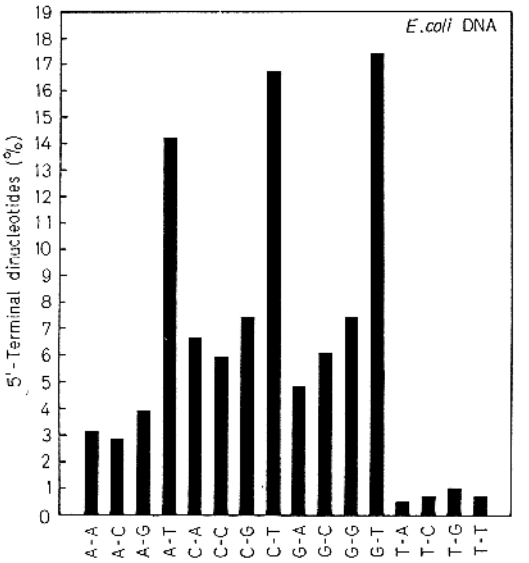

Fig. 5. *5'-Terminal dinucleotides released by crab DNAase from* E. coli *DNA*

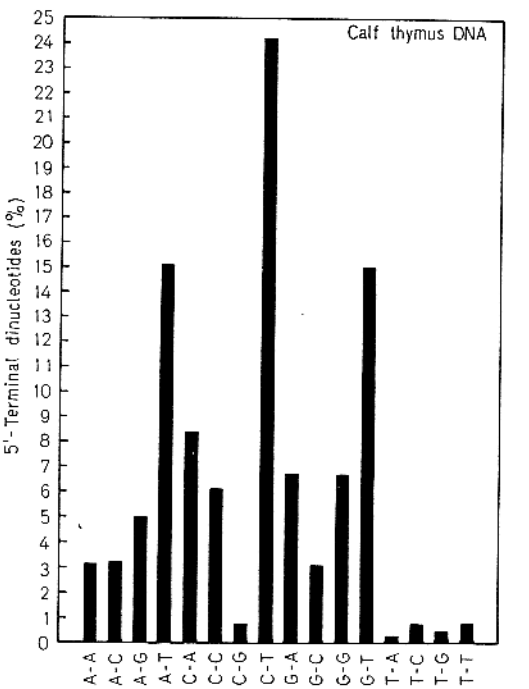

Fig. 6. *5'-Terminal dinucleotides released by crab DNAase from calf thymus DNA*

mononucleotides, and by the presence of a large amount of unresolved material. In this latter respect, the crab enzyme is similar to spleen [7] and snail DNAases [9], and different from pancreatic [10] and *E. coli* DNAases [11].

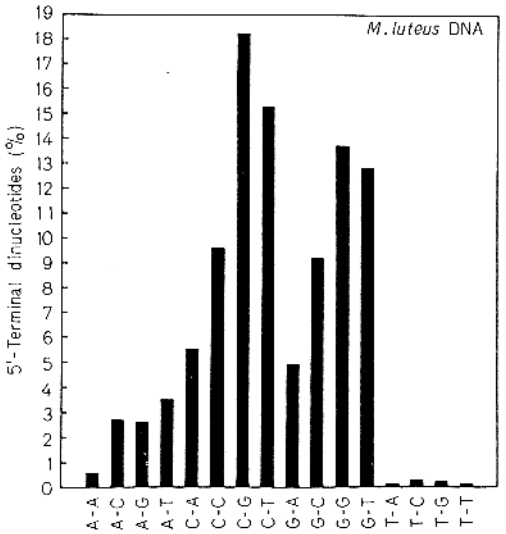

Fig. 7. *5'-Terminal dinucleotides released by crab DNAase from* M. luteus *DNA*

Specificity

The specificity of the crab DNAase certainly is the outstanding feature of this enzyme. In the case of a compositionally equilibrated DNA, like *E. coli* DNA (51% dG + dC), the digest is characterized by a very large amount of thymidine in both the 5'-penultimate (≈ 50%) and 3'-terminal position (37%) and by an almost complete absence of thymidine in the intermediate, 5'-terminal position (≈ 1%). The 5'-terminal dinucleotide histogram is characterized by the predominance of thymidine-ending doublets and by the practical absence of thymidine-starting doublets. This result is important in several ways. First of all, it directly shows that the enzyme recognizes and splits sequences at least three-nucleotides long; the composition of termini is so peculiar that in this case a check on this statement using nearest-neighbor data (such as it was done previously for other DNAases [7, 9–12]) is superfluous. Second, it is particularly encouraging in so far as a screening of DNAase specificity is concerned. More particularly, the specificity of the crab enzyme, as well as that of the snail enzyme [9] suggests that invertebrate DNAases may be particularly rewarding in this respect, perhaps because of the large phylogenetic distances among invertebrates belonging to different classes. Finally, it should be pointed out that the crab enzyme is the most specific animal DNAase investigated so far, and probably ranges second in specificity only to the T4-induced endonuclease IV [14]. Further comments on the specificity of the crab enzyme are presented in the accompanying paper [15].

The results obtained on the 5'-terminal doublets released from *M. luteus* and calf thymus DNAs cannot be commented in detail at the present time, since this would need among other things, a knowledge of the variation of 5'-terminal dinucleotides with changing base composition in bacterial DNAs [1]. They are shown, however, because of some indications they can give. The *M. luteus* data show the striking changes accompanying the high dG + dC contents (72%) of this DNA. The calf thymus results are interesting in another respect; in this case, the difference in dG + dC contents from the *E. coli* DNA is not nearly as important as for *M. luteus* DNA, since calf thymus DNA is 44% in dG + dC, but repetitive sequences are present. The most interesting results concern the very low level of dG-dG, related to the shortage of this doublet in vertebrate DNAs [16], and the very high level of dC-dT.

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