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# STRUCTURE, FUNCTION AND EVOLUTION OF *Serratia marcescens* ENDONUCLEASE

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**ABSTRACT**

The Gram-negative enterobacterium *Serratia marcescens* produces a variety of hydrolases that are secreted into the surrounding medium, among them some are highly active DNA/RNA nonspecific endonuclease. This nuclease has been the focus of studies on its mechanism of action, its substrate preferences, its protein structure and its application in industrial biotechnology. Up to date several closely and more distantly related nucleases are known that together form a *Serratia* nuclease superfamily. Here we briefly review these different aspects of research regarding the work on *Serratia* nuclease.

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## 1 Introduction

The Gram-negative bacterium *Serratia marcescens* secretes a variety of hydrolases, among them nuclease (Eaves & Jeffries, 1963; Nestle & Roberts, 1969), several proteases (Bromke & Hammel, 1979; Braun & Schmitz, 1980), lipases and phospholipases (Heller, 1979; Givskov et al., 1988; Li et al., 1995), as well as chitinases and chitobiases (Monreal & Reese, 1969; Jones et al., 1986) are most common one and these hydrolytic enzymes allow this soil bacterium to "digest" whole organisms and their remnants, in particular those of fungi and insects, and to convert complex structures into simple metabolites. Among these extracellular enzymes, *Serratia* nuclease is most studied one and it is capable to cleavage both RNA and DNA in either single or double stranded form, with little sequence preference (Eaves & Jeffries, 1963). At the same time it is one of the most active nucleases known, which only requires Mg2+ (Mn2+, Co2+, or Ni2+) as cofactor, and has a broad pH and temperature optimum, and displays a pronounced stability towards detergents and chemical denaturants (Eaves & Jeffries, 1963; Nestle & Roberts, 1969; Yonemura et al., 1983; Biedermann et al., 1989).

*Serratia* nuclease is the product of the *nucA* gene whose expression is growth phase regulated (Chen et al., 1992; Chen et al., 1995) and involves stimulation of transcription by the *nucC* gene product which binds to a region upstream of the transcriptional start site of the *nucA* gene (Jin et al., 1996). In addition, *nucA* gene expression increases during the SOS response involving RecA-stimulated autoproteolysis of LexA which has binding sites upstream of the *nucA* and *nucC* genes (Ball et al., 1990; Chen et al., 1992; Jin et al., 1996).

*Serratia* nuclease is produced as a pre-protein of 266 amino acids with an N-terminal signal peptide consisting of 21 residues (Ball et al., 1987). Cleaving off the signal sequence yields two major isoforms, Sml (242 amino acids) and Sm2 (245 amino acids), which are also produced in recombinant *Escherichia coli* strains (Filimonova et al., 1991). It is believed that these isoforms are the products of alternative cleavage of the pre-proteins by the signal peptidase, Sm2 being formed predominantly during exponential growth and secreted extracellularly and Sm1 being produced in the stationary phase and remaining more or less trapped in the periplasm (Bannikova et al., 1991). Otherwise, Sm1 and Sm2 have very similar biochemical properties (Bannikova et al., 1991; Pedersen et al., 1993a; Pedersen et al., 1993b). Minor isoforms have been detected by capillary electrophoresis (Pedersen et al., 1993c) and electrospray mass spectroscopy (Pedersen et al., 1995); the physiological significance of the formation of these isoforms is not known.

Upon secretion of *Serratia* nuclease into the oxidizing milieu of the periplasm two disulfide bonds (C19/C13 and C201/C243) are formed (Pedersen et al., 1993a) which are essential for the stability and activity of the nuclease (Ball et al., 1992; Schofield et al., 2017). The inactivity of the reduced form of *Serratia* nuclease explains why *S. marcescens*, unlike *Anabaena* (vide infra), does not require an intracellular inhibitor for a potentially highly toxic protein.

Earlier work had shown that the *Serratia* endonuclease that in the presence of Mg2+ cleaves single and double stranded RNA and DNA with similar activity and produces 5'-phosphorylated (mono-), di-, tri- and tetranucleotides (Eaves & Jeffries, 1963; Nestle & Roberts, 1969), but the mechanism of phosphodiester bond hydrolysis was not known. It is well established that *Serratia* nuclease is a nonspecific endonuclease, but like other nonspecific nucleases it also need preferences of certain substrates for cleavage. For example, while poly (I) • poly(C) is cleaved by this enzyme as readily as natural DNA and RNA, poly(dA) • poly(dT) is largely resistant to cleavage (Yonemura et al., 1983). Similarly, pyrimidinic portion of DNA cleaved in better manner than a purinie portion of DNA (Balaban et al., 1971, Balaban & Leshchinskaya, 1971). No explanation existed for the structural basis of these preferences. The lack of mechanistic knowledge was surprising, as *Serratia* nuclease is still as an enzyme of major commercial importance: under the trade name Benzonase it is used for the downstream processing in large scale of biochemical and pharmaceutical products. One reason for the fact that so little was known regarding mechanistic details presumably was due to the absence of structural information, other than the sequence (Ball et al., 1987; Biedermann et al., 1989; Burritt et al., 2016; Rai & Adams, 2016). The main purpose of this work was to unravel the mechanism of phosphodiester bond hydrolysis by *Serratia* nuclease, to understand the substrate preferences of this enzyme, and to find out whether *Serratia* nuclease can be used as a biotechnological tool to detect and remove nucleic acids in biochemical and pharmaceutical preparations. To this end a detailed biochemical characterization of this enzyme was required.

## 2 The mechanism of phosphodiester bond hydrolysis by *Serratia* nuclease

Identification of *Serratia* nuclease active site was began with a mutational analysis which concentrating on residues that were conserved in related enzymes isolated from *Serratia marcescens*, *Anabaena* sp. (Muro-Pastor et al., 1992), *Saccharomyces cerevisiae* (Vicent et al., 1988), and *Bos taurus* (Ruiz-Carrillo & Cote, 1993). For the mutational analysis, the *nucA* gene was cloned into an over expression vector which allowed to produce recombinant *Serratia* nuclease in yields of over 10 mg / 500 ml

*E.coli* culture (Friedhoff et al., 1994a). To facilitate purification, the *nucA* gene was fused subsequently to a sequence coding for a His6-tag, and the mutational analysis was carried out with His6-tagged variants. The alignment had identified several conserved residues among the four proteins, some of which proved to be essential for catalysis (Friedhoff et al., 1994b), in particular His89 and Glul27 which in the crystal structure, determined at the same time (Miller et al., 1994), turned out to be located close in space and such that they could be considered as being directly involved in catalysis (Figure 1). Based on the detailed structural data and a refined alignment of six related nucleases, including *Syncephalostrum racemosum* (Miller et al., 1994) and *Streptococcus pneumoniae* (Puyet et al., 1990), candidate amino acid residues conserved among these six proteins, located close to His89 and Glul27, as well as likely to fulfill a catalytic function, were conservatively substituted and the resulting nuclease variants tested using in part a newly developed time saving quantitative microtiter plate assay (Friedhoff et al., 1996a).

The steady-state kinetic analysis, which included determination of the pH and metal ion dependence of the nucleolytic activity of the variants towards different DNA and RNA substrates, allowed to put forward a reasonable model for the mechanism of phosphodiester bond cleavage by *Serratia* nuclease (Friedhoff et al., 1996b). According to this model, His89 is the general base that serves to activate a water molecule for an in-line attack on the phosphodiester bond. An alternative candidate for this function, Glu127, was ruled out by the results of a study in which a minimal substrate with a good leaving group was used, deoxythymidine 3’5'-bis-(p-nitrophenyl phosphate). This substrate was cleaved by the E127A variant but not by the H89A variant, indicating that Glu127 could be involved in leaving group stabilization but not in deprotonating the attacking water molecule (Kolmes et al., 1996). In the meantime the structure of *Serratia* nuclease with a Mg2+ ion bound to the active site had been determined (Miller et al., 1999). Together with the results of the afore mentioned site-directed mutagenesis experiments a detailed mechanism of phosphodiester bond hydrolysis can now be formulated. Recently, independent evidence for the correctness of this mechanism came from a comparison of the structures of *Serratia* nuclease (Miller et al., 1994; Lunin et al, 1997) and the homing endonuclease *I-PpoI* (Flick et al., 1998) which showed that these enzymes, one being a nonspecific nuclease and the other one a nuclease of extreme specificity, share a common active site architecture (Friedhoff et al., 1999a) and can cleave the same artificial substrate, deoxythymidine 3',5'-bis-(-*p*-nitrophenyl phosphate) (Friedhoff et al., 1999b).

**3 Substrate preferences of the *Serratia* nuclease**

In parallel with the steady-stale kinetic analysis of the cleavage of high molecular weight DNA by *Serratia* nuclease variants, also cleavage of other natural as well as synthetic nucleic acid substrates by the wild type enzyme was analyzed (Meiss et al., 1995; Friedhoff et al., 1996a). The results of various studies suggested that *Serratia* nuclease is indeed a very efficient enzyme,

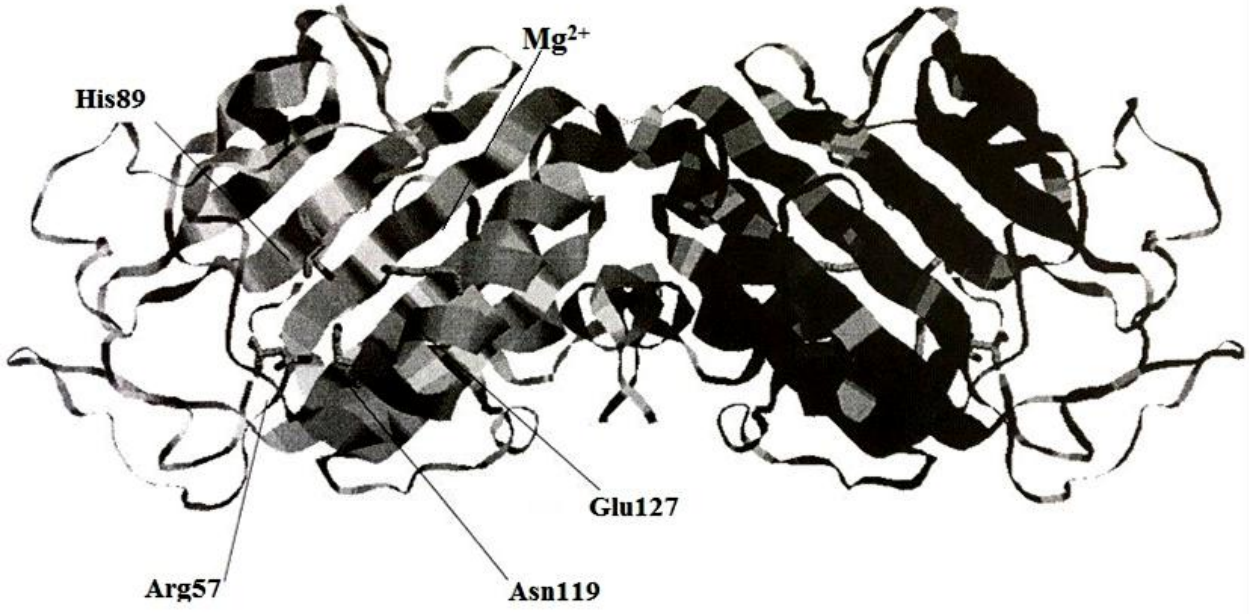


Figure 1 Structure of the Serratia nuclease dimer with $Mg^{2+}$ [39]. The active site residues Arg57, His89, Asn119, Glu127 and the $Mg^{2+}$ with 5 coordinating water ligands are shown. Note that of the the two active sites are located on opposite sides of the nuclease. Right bottom: blowup of the active site side chains.

cleaving natural DNA about three times faster than *Staphylococcus* nuclease and 30 times faster than DNase I (Friedhoff et al., 1996a). In natural DNA, it shows preferences for G+C-rich regions, in particular (dG) •(dC) tracts, and avoids cleavage of (dA) • (dT) tracts (Meiss et al., 1995). Accordingly, poly(dG) • poly(dC) is cleaved more than 50 times faster than poly- (dA) •poly(dT) (Friedhoff et al., 1996a). Preference for double stranded nucleic acids in A-form, as shown the finding that poly(A) •poly(U) is cleaved almost 20 times faster than poly d(A) • poly(dT) (Friedhoff et al., 1996a), and by the detailed comparison of the rates of cleavage of synthetic single- and double-stranded oli- goribo- and oligodeoxyribonucleotides as well as defined RNA transcripts and DNA fragments (Meiss et al., 1999). It demonstrated that *Serratia* nuclease prefers the A- form over the B-form makes sense in evolutionary terms: the nucleic acid substrate that *Serratia marcescens* will encounter in its natural habitat is partially double-stranded RNA and to a lesser extent DNA. While double-stranded RNA adopts the A-form, double-stranded DNA can occur in the A- or the B-form depending on the milieu, in particular the water activity. In the soil, the natural habitat of *Serratia marcescens*, conditions are likely to be such that the A-form is the favored conformation of double-stranded DNA. In addition to preferences for a certain "global" structure, "local" features of a sequence influence the rate of cleavage. Two conserved aromatic amino acid residues (Tyr76 and Trp123) close to the active site arc not responsible for the influence of "local" features on the rate of cleavage at particular sites, as could have been expected (Meiss et al., 1999). It was expected, therefore, that it is the total architecture of the substrate binding site that determines what a preferred site must look like, which means that preferential cleavage is not due to the preferential interaction between the substrate and one particular amino acid residue (Pchelintsev et al., 2016).

## 4 Quaternary structure-function relationships among nucleases of the *Serratia* nuclease family

*Serratia* nuclease is a homodimer (Friedhoff et al., 1994b; Filimonova et al., 1981; Miller & Krause, 1996; Franke et al., 1998), while the related *Anabaena* nuclease is a monomer (Meiss et al., 1998). The question arises, what the consequences of being a dimer for *Serratia* nuclease are. To approach this problem, monomeric and obligatory dimeric versions of *Serratia* nuclease were constructed and their activities compared with each other and with the *Anabaena* nuclease. To produce monomeric variants, His184, which is located at a critical position in the dimer interface of the *Serratia* nuclease dimer (Miller & Krause, 1996), was substituted by other amino acid residues, e.g. Arg. resulting in a perfectly soluble, stable monomeric variant (Franke et al., 1998). An obligatory dimeric variant was obtained by first introducing a Cys residue in place of Ser140 and then cross-linking the two subunits via Cys140 using bismaleimidoalkanes (Franke & Pingoud, 1999). The monomeric and the obligatory dimeric variants display the same specific activity (normalized to the concentration of active sites) as the wild type enzyme, demonstrating that the two subunits in wild type *Serratia* nuclease function independently to each other; however, at very low enzyme and substrate concentrations dimeric forms of *Serratia* nuclease are relatively more active than monomeric forms or the naturally monomeric *Anabaena* nuclease toward high molecular weight nucleic acid substrates (Franke et al., 1999). This is correlated with the ability of dimeric forms of the *Serratia* nuclease to form large enzyme-substrate networks with high molecular weight DNA and to cleave polynucleotides in a processive manner (Franke et al., 1999). The advantage for *Serratia marcescens* of having a dimeric endonuclease, it is more efficient in utilization of extracellular nucleic acids as precursors for nucleotide metabolism and as source for carbon, nitrogen and phosphorous (Beliaeva et al., 1976), when these are growth-limiting in the environment.

## 5 Similarities between *Serratia* nuclease and closely and distantly related enzymes

The *Serratia* family of nucleases is characterized by the signature motif DRGH (prosite motif PDOC00821) which contains the catalytically essential His residue. It currently consists of 16 members (Figure 2), which occur in prokaryotic as well as eukaryotic organisms including humans. These enzymes fulfill different cellular functions: prokaryotic enzymes seem to serve mainly nutritional purposes, while eukaryotic enzymes are involved in mitochondrial DNA replication (Ruiz-Carrillo & Cole, 1993) and repair (Dake et al., 1998). One of the best studied enzymes of the *Serratia* nuclease family, other than *Serratia* nuclease itself, is the *Anabaena* nuclease (Muro-Pastor et al., 1992) present in many species of the genus *Anabaena* (Muro-Pastor et al., 1997). Like the *Serratia* nuclease, it is secreted from its host organism, but different from *all* other members of the *Serratia* nuclease family, it is produced together with a polypeptide inhibitor that is specific for the *Anabaena* nuclease and effectively blocks any intracellular activity of this enzyme (Muro-Pastor et al., 1997; Meiss et al., 1998). *Anabaena* and *Serratia* nuclease share 30% sequence identity. The *Anabaena* enzyme, however, does not have disulfide bridges and is a monomer. Otherwise, it has very similar catalytic properties (Meiss et al., 1998; Meiss et al., 2000). This fact that amino residues involved in substrate binding and phosphodiester bond hydrolysis by *Serratia* nuclease are conserved in the *Anabaena* enzyme, suggest that both enzymes may follow the same mechanisms of action. Indeed, substitutions of these amino acid residues by Ala led to variants which are similarly affected as the corresponding *Serratia* nuclease variants: R93A (R57A), D121A

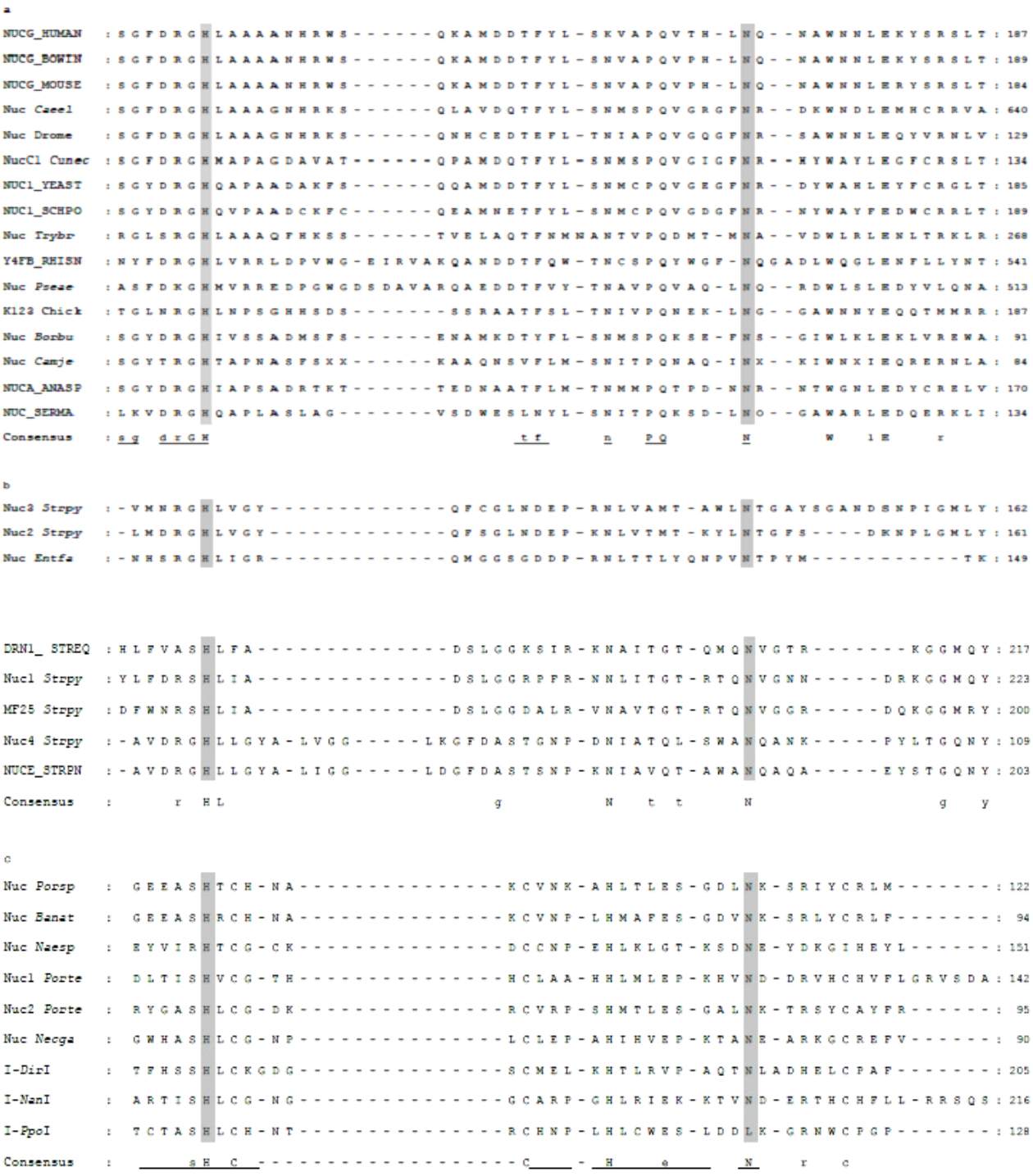


Figure 2 Alignment of the presumptive active site regions of three different families of nucleases. Protein sequence alignment of the family of a DNA/ RNA nonspecific endonucleases, b nucleases homologous to the DNA-entry nuclease of *Streptococcus pneumonia,* and c the Cys-His box containing nuclear homing endonucleases. The catalytically relevant histidine and asparagine residues are shown in grey background. Note, the alignment in b has been edited to optimize the sequence homology with a, while the alignment in c has been optimized based on structural similarity *Serratia* nuclease and I-*Ppo*I in the active site region. The structurally superimposable residues in *Serratia* nuclease and I-*Ppo*I are indicated by a horizontal line within the consensus sequences.

(D86A), H124A (H89A), R122A (R87A), N151A (N119A), E163A (E127A), R167A (R131A) (Meiss et al., 2000). It can be conclude that *Anabaena* nuclease follows the mechanism of DNA cleavage as *Serratia* nuclease is strengthened by the finding that *Anabaena* nuclease cleaves the artificial minimal substrate thymidine 3',5'-bis- (p-nitrophenyl phosphate) (Meiss et al., 2000).

While the sequence similarities are sufficiently high between the members of the *Serratia* nuclease family to suppose that they have a similar three-dimensional structure, this is not the case for other nucleases that have a similar catalytic sequence motif, the prime example being the homing endonuclease *I-PpoI*. The co-crystal structures of the *I-PpoI* -substrate and *I-PpoI* -product complexes were determined recently (Flick et al., 1998). *I-PpoI* is an extremely specific DNase that recognizes and cleaves the two strands ol a palindrome 14 base pair sequence, while the *Serratia* nuclease is a nonspecific nuclease that cleaves RNA and DNA, in single and double stranded form. Along with these differences in function and overall structure, these two share a common catalytic core motif (Friedhoff et al., 1999a). Furthermore, both are able to cleave the artificial substrate thymidine 3',5'-bis-(*p*-nitrophenyl phosphate) (Friedhoff et al., 1999b).

The similarity of the structure of the catalytic cores of *Serratia* nuclease and *I-PpoI* is hardly at all reflected in the amino acid sequences of these proteins (Figure 2). Only the general base (His 89 in *Serratia* nuclease and His 98 in *I-PpoI)* and the Mg-ion ligand (Asn 119 in both enzymes) are conserved. Nevertheless, given the structural and mechanistic similarities and the results of site-directed-mutagenesis experiments, there is no reasonable doubt that these enzymes share a common mechanism for phosphodiester bond hydrolysis. In addition, the structure of the *I-PpoI* -substrate complex allows drawing conclusions as how nucleic acid substrate could be bound by *Serratia* nuclease, which information not yet available, as a co-crystal structure of a *Serratia* nuclease-substrate complex has not been determined so far. If the structures of the *I-PpoI* -DNA complex and *Serratia* nuclease are superimposed, the DNA bound to *I-PpoI* is not clashing into *Serratia* nuclease, but rather fits smoothly into the active site of this enzyme (Figure 3). In this model three phosphate residues make contact to the three Arg residues (Arg 57, Arg 87 and Arg 131). Both these phosphate and these Arg residues were demonstrated previously by chemical modification studies and by a mutational analysis to be required efficient cleavage (Friedhoff et al., 1996a; Friedhoff et al., 1996b; Friedhoff et al., 1996c; Srivastava et al., 1999) demonstrating that the model is not unreasonable.

The comparison of the structures of the nonspecific *Serratia* nuclease and the homing endonuclease *I-PpoI* have allowed identifying a common catalytic core motif for these two enzymes and their homologues, in spite of the absence of significant sequence homologies. The sequence information for the two only distantly related families of nonspecific nucleases on one side and homing endonucleases on the other side has been used to search for other distantly related nucleases. So far, a third family of nuclease has been identified, the DNA-entry nuclease family (Figure 2) that shares the catalytic core motif with the *Serratia* nuclease family and the Cys-His box family of homing endonucleases (Friedhoff et al., 1999b), to which *I-PpoI* belongs. While a detailed mutational analysis has not yet been carried out for any DNA-entry nuclease, it has been shown for one of them, the mitotic factor nuclease of *Streptococcus pyogenes*, that His122 is essential for catalysis (Iwasaki et al., 1997). Thus it seems as if the mechanism of phosphodiester bond cleavage is not unique for the members of the *Serratia* nuclease family but is also used by other nucleases, nonspecific as well as highly specific ones. Given the little sequence homology among the different families of nucleases and the absence of overall structural similarity of *Serratia nuclease* and *I-PpoI*, it is reasonable to assume that these families have evolved independently of each other and that the similarities in their active sites are the outcome of convergent evolution.

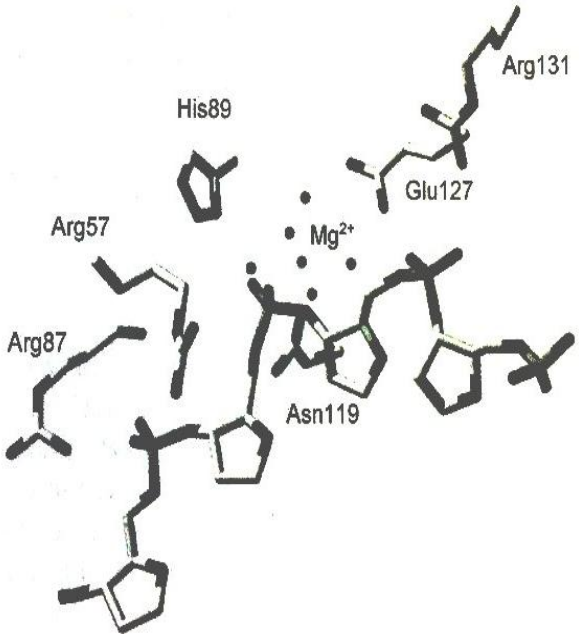


Figure 3 A model for the substrate binding site of Serratia nuclease. The active site residues Arg57, His89, Asn 119, Glu127 and the $Mg^{2+}$ with 5 coordinating water ligands as well as two residues, namely Arg87 and Arg131, likely to be involved in substrate binding are shown. The position of the DNA (for clarity only the phosphodiester backbone of a tetranucleotide piece of DNA is shown) is obtained from the I-*Ppo*I-DNA co-crystal structure after superposition of the active sites of I-*Ppo*I and *Serratia* nuclease.

**6 Future aspects regarding *Serratia* nuclease**

When *Serratia* nuclease studies were begun the main goal was to understand how this remarkably efficient enzyme works, the goal that has been achieved, in particular, because structural information became available. Over the last years, the interest in the enzymology of Serratia nuclease shifted in part to other related enzymes, among them the Anabaena nuclease, for which it is important to know how this enzyme interacts with its inhibitor.

*Serratia* nuclease as a nonspecific and highly active enzyme is a very interesting biotechnological tool. Immobilized on solid support it could be used to remove nucleic acids from biochemical and pharmaceutical preparations, or to constitute the bio-component in a biosensor for the potentiometric detection of nucleic acids for many purposes. So far, a stable immobilization with high yield and preservation of activity has not been achieved, presumably because spacers were not sufficiently long to allow macromolecular nucleic acid substrates to approach the active site of the enzyme.

**Conflict of interest**

Authors declare that no conflict of interest could arise

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