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A non-specific nucleolytic enzyme and its application potential in EDTA-containing buffer solutions

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Abstract
Objectives Metal-ion independent non-specific nucleases are of high potential for applications in EDTA-containing bioprocessing workflows.
Results A novel extracellular non-specific nuclease *Ec*Nuc from the enterobacterium *Escherichia coli* has been identified. The recombinant gene was expressed and the protein was purified. Maximum activity of the enzyme was detected at 41.7 °C and at an acidic pH of 5.8. *Ec*Nuc tolerates EDTA in the reaction buffer at concentrations of up to 20 mM and the activity is not impaired by high concentrations of mono- and divalent metal ions in the absence of EDTA. The viscosity of crude protein extracts after cell lysis in EDTA-containing buffers is reduced when supplemented with *Ec*Nuc.
Conclusion Proof-of-concept has been demonstrated that a metal-ion independent non-specific nuclease can be applied for removal of nucleic acids in EDTA-containing buffers for the subsequent purification of proteins from crude extracts.

Keywords Biotechnological application · DNase · EDTA · RNase

Introduction

Nucleases are ubiquitous in pro- and eukaryotic organisms. Their roles comprise the utilization of DNA as nutrients, they are involved in biofilm degradation or they act as a barrier to transformation of (host) cells by nucleic acids (Brown et al. 2012; Rangarajan and Shankar 2001). Due to their catalytic activity, nucleases are highly demanded in biotechnology and molecular biology approaches. Specific nucleases are widely applied as restriction enzymes, while non-specific nucleases are mainly utilized for the degradation of nucleic acids in crude protein extracts to decrease viscosity and to remove contaminating host cell nucleic acids during protein purification (Rangarajan and Shankar 2001).

Nucleases are classified based on primary sequences, sequence specificities, reaction mechanisms, substrates and reaction products (Yang 2011). Most non-specific nucleases are metal-ion dependent enzymes with NucA from *Serratia marcescens* being the best characterized isozyme. In this nuclease, a magnesium ion is obligatory and mainly coordinated by Asn119 acting as the principle ligand, while His89 is a general base that activates a water molecule for a

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nucleophilic attack on the phosphorous ester in the nucleic acid backbone (Benedik and Strych 1998). NucA from *S. marcescens* is commercially sold (trademark "Benzonase® Nuclease") to be used in downstream processing, mainly for the elimination of nucleic acid contaminations in protein purification processes (Rangarajan and Shankar 2001).

There is only a limited number of nucleases known that do not require divalent metal ions for their catalytic activity including the restriction enzyme *Lla*KI from *Lactococcus lactis* KLDS4, *GBSV1-NSN* from the thermophilic bacteriophage GBSV1 and R.PabI, which is encoded by transposable elements in Archaea (Belkebir and Azeddoug 2012; Song and Zhang 2008; Wang et al. 2016). The non-specific DNase II-like proteins isolated from parasitic nematodes and the *Bfi*I-type restriction enzymes from members of the genus *Bacillus* can be assigned to the phospholipase D family (Bao et al. 2008; Liao et al. 2014; Yang 2011). This superfamily of proteins is defined by a common catalytic domain represented by a typical $HxK(x)_4D(x)_6GSxN$ motif that has been structurally characterized for the endonuclease Nuc from *Salmonella enterica* subsp. *enterica serovar Typhimurium* (Stuckey and Dixon 1999). Bacterial nucleases exhibit a single motif, while related proteins from eukaryotes are characterized by two copies of the consensus sequence. This group of enzymes is not only restricted to nucleases, but also contains phosphatidylserine and cardiolipin synthases and a *Yersinia* murine toxin (Rudolph et al. 1999; Stuckey and Dixon 1999).

In this study, a distantly related homologue of Nuc was identified in *Escherichia coli*. The gene was cloned and expressed and the recombinant protein was purified. Biochemical properties are reported and the potential application of *Ec*Nuc in downstream processing with a focus on EDTA-containing buffers was investigated.

Methods

Computational analysis

BLASTP analyses were used to identify uncharacterized nucleases in bacterial genomes. A putative endonuclease from *Escherichia coli*, which is deposited under the accession no. KZO82453.1, was identified and the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict a hydrophobic signal peptide sequence. GraphPad was used for curve fitting purposes (Motulsky 2016).

Gene cloning, expression and purification

Culture conditions, plasmid propagation and maintenance as well as transformation of bacterial cells were done using standard molecular biology techniques. Plasmid pET-24d(+) in combination with *Escherichia coli* Veggie BL21 (DE3) Singles (both Merck, Darmstadt, Germany) was used for gene expression purposes of a signal peptide free and codon optimized gene variant (ATUM, California, USA) (Supplementary Figure 1). The recombinant protein was produced as a double 6xHIS-tag fusion enzyme (Fig. 1a) and purified in a three-step purification process: (1) affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany), (2) ion exchange chromatography using SP-Sepharose (GE Healthcare, Solingen, Germany), (3) proteolytic cleavage followed by another affinity chromatography using Ni^{2+}-NTA agarose (Fig. 1b, c). The following buffers were used for purification approaches: (1) 50 mM $NaPO_4$, 50 mM NaCl, pH 7.0 (equilibration buffer), plus 150 mM imidazole (washing buffer), or plus 500 mM imidazole (elution buffer), (2) 25 mM $NaPO_4$, pH 6.0 (equilibration buffer), plus 300 mM NaCl (washing buffer), or plus 1000 mM NaCl (elution buffer), (3) flow-through containing non-tagged nuclease was sampled. Finally, buffer exchange was done using PD-10 columns (GE Healthcare, Solingen, Germany) and the protein was stored in 25 mM $NaPO_4$, 25 mM NaCl, pH 7.0. A semi-dry Western blotting system (Biometra, Göttingen, Germany) was used to transfer proteins onto Roti®-PVDF membrane (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). An anti-His-HRP antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) was used in combination with the Immobilon™ Western HRP substrate (Merck, Darmstadt, Germany). Identity of the purified protein was verified by peptide mass fingerprinting (PMF) (data not shown).

Enzyme activity assays

Qualitative assays: about 100–200 ng of purified enzyme was incubated for 30 min in reaction buffer

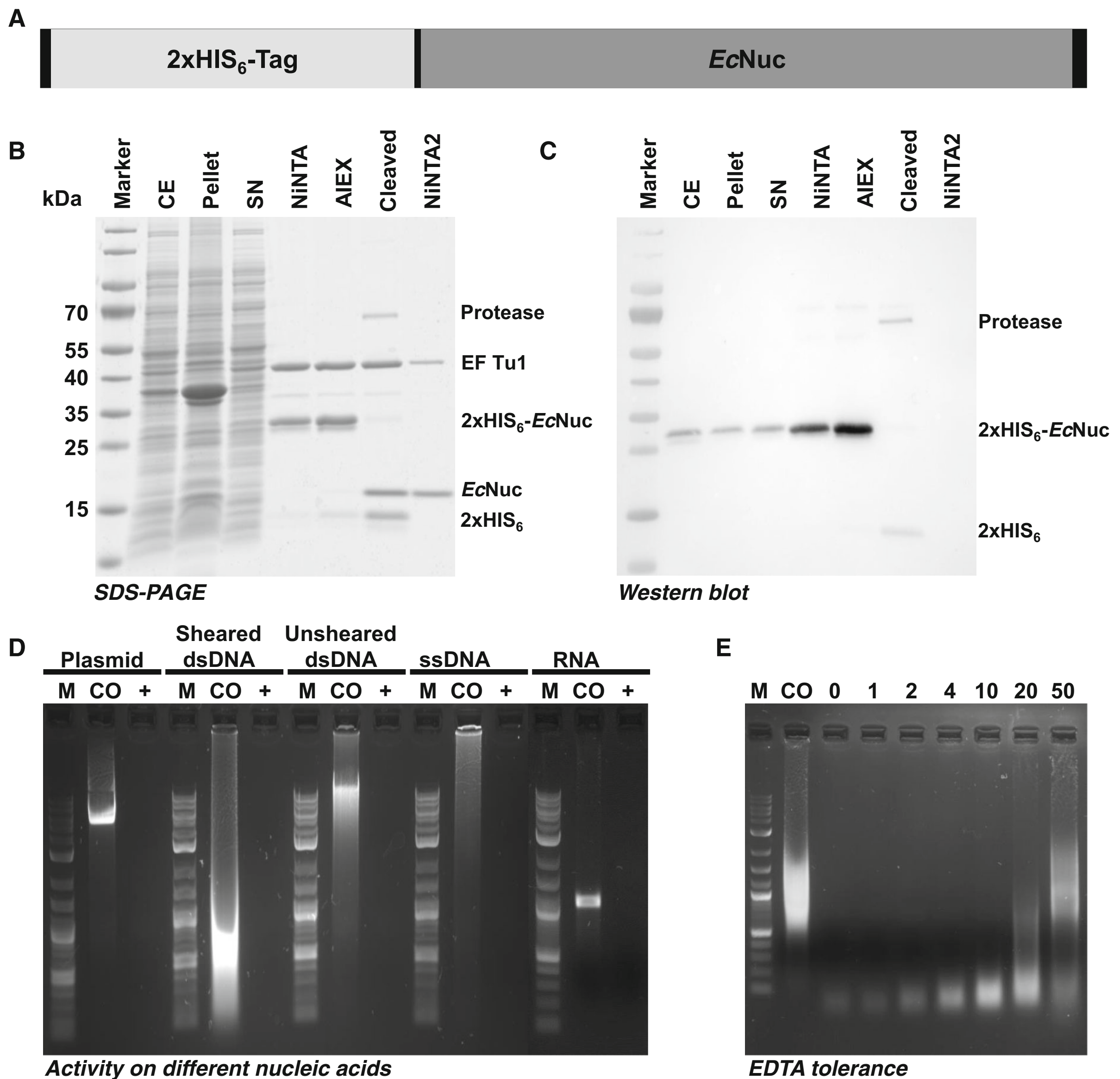

Fig. 1 Production, purification, substrate specificity and EDTA-tolerance of the non-specific nuclease *Ec*Nuc. **a** Schematic illustration of the fusion protein 2xHIS$_6$-*Ec*Nuc construct including twin 6xHIS tag, protease cleavage site and coding region of *Ec*Nuc. SDS-PAGE (**b**) and Western blotting analyses (**c**) of samples from cell extract to purified *Ec*Nuc. *E. coli* Veggie BL21 (DE3) cells harboring the plasmid pET24d(+)::2xHIS$_6$-*Ec*Nuc were grown for 20 h without induction at constant shaking and 37 °C (CE—crude extract). Induction of expression led to lethality. *SN* supernatant, *Pellet* pellet fraction, *NiNTA* eluted fraction after affinity chromatography, *AIEX* eluted fraction after anion exchange chromatography, *Cleaved* proteolytically cleaved fusion proteins, *NiNTA2* flow through of untagged *Ec*Nuc after affinity chromatography. **d** To determine the substrate specificity, *Ec*Nuc was incubated with different types of DNA. The degradation efficiency towards the expression plasmid pET24d(+)::2xHIS$_6$-*Ec*Nuc (Plasmid) was compared to unsheared dsDNA from calf thymus, to sheared dsDNA from salmon sperm, to ssDNA and to RNA, respectively. **e** Activity in EDTA-containing buffer was analyzed at concentrations between 1 and 50 mM EDTA. All assays were performed in 50 mM sodium phosphate buffer, pH 6.0 at 40 °C

supplemented with DNA at a concentration of 5 µg in a final volume of 20 µl. Protein concentrations were determined with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). Reaction was stopped by the addition of 0.25% (w/v) sodium dodecyl sulfate (SDS) prior to agarose gel analysis and ethidium bromide staining. Assays were performed in 50 mM sodium phosphate buffer at pH 6.0 and 25 °C, while concentrations of detergents, metal ions and chelators were varied. Substrate specificity was tested using circularized plasmid DNA, single-stranded DNA (ssDNA) from calf thymus (Sigma-Aldrich, St. Louis, USA) and double-stranded sheared and unsheared genomic DNA (dsDNA) namely UltraPure™ Salmon Sperm DNA Solution (Thermo Fisher Scientific, Darmstadt, Germany), deoxyribonucleic acid from calf thymus, and MS2 RNA (both Sigma-Aldrich, St. Louis, USA).

Quantitative assays: about 20 ng of purified enzyme was incubated for 10–30 min in reaction buffer supplemented with DNA at a concentration of 0.5–60 µg in a final volume of 100 µl. The formation of acid-soluble nucleotides was determined spectrophotometrically at 260 nm in the VICTOR™ X4 Multilabel Plate Reader. The nucleolytic degradation of plasmid DNA was measured in a continuous approach, while enzymatic activity towards genomic DNA (single- and double-stranded, sheared and unsheared) was determined by end point measurements. One unit of nuclease activity is defined as ΔA_{260} min^{-1} per mg protein. A mean molar mass per nucleotide of 330 g mol^{-1} was defined to calculate µM from concentrations of sheared dsDNA. k_{cat} values were calculated from v_{max} by the assumption that the degradation of 0.05 mg DNA resulted in a change in absorbance of 0.3 in combination with the general formula $k_{cat} = V_{max}/[E_0]$ (MacLellan and Forsberg 2001).

To evaluate the application potential of *Ec*Nuc for the elimination of nucleic acids from crude protein extracts, *E. coli* Veggie BL21 (DE3) Singles were transformed using the mock vector pET24d(+) and grown to high cell densities (data not shown). Afterwards, cells were harvested and resuspended in 50 mM sodium acetate buffer, pH 6.0 prior to cell lysis (High-pressure homogenization). One gram of cells were dissolved in 4 ml of buffer. Lysed cells were either supplemented with *Ec*Nuc (~ 10 Units) or with a metal-ion dependent nuclease (Benzonase® Nuclease, Merck, Darmstadt, Germany, ~ 10 Units) and incubated with or without 20 mM EDTA for 1 h at 35 °C. Viscosity of protein extracts was tested by gravity flow experiments using 1.2 ml pipette tips (Eppendorf, Wesseling-Berzdorf, Germany).

Results

A putative endonuclease from *Escherichia coli*

BLASTP analyses using a characterized endonuclease of the phospholipase D (PLD) family from *Salmonella enterica* subsp. *enterica serovar Typhimurium* (formerly known as *Salmonella typhimurium*, Nuc, PDB: 1BYS_A) as input sequence revealed the presence of a distantly related homologue in the enterobacterium *Escherichia coli* (KZO82453.1). The unprocessed protein is composed of 169 amino acid residues with a theoretical isoelectric point of pI 9.2 and a predicted molecular mass of 18.4 kDa. A putative signal peptide for secretion into the periplasmic space was predicted to be covered by amino acid residues 1–16. The sequence identity of *Ec*Nuc to Nuc is 65.3%. A typical sequence motif of the PLD superfamily $HxK(x)_4$-$D(x)_6GSxN$ is conserved between His^{108} and Asn^{125} (Stuckey and Dixon 1999).

Purification of *Ec*Nuc

Expression of the gene in *E. coli* as fusion protein composed of a $2xHis_6$ tag, a linker including protease cleavage site and the codon-optimized gene encoding *Ec*Nuc without the signal peptide and purification of the recombinant protein was visualized by Coomassie-stained SDS gel and Western blotting analysis using anti-His-HRP antibody. A single band of ~ 30 kDa was detected by Western blot that is conform to the calculated mass of the *Ec*Nuc fusion protein (12 + 17 kDa). A cleaved variant that is exclusively composed of the mature enzyme is visible on the SDS gel (Fig. 1b), but not detectable by Western blot analysis (Fig. 1c). A second band of ~ 42 kDa on the SDS gel has been identified by PMF to be *E. coli* elongation factor Tu1 (data not shown). A final yield of 39% and a purification factor of 171 was obtained for *Ec*Nuc (Supplementary Table 1). About 1.5–2.0 mg purified protein was obtained from 1 l high cell density fermentation (data not shown).

Catalytic activity of *Ec*Nuc

*Ec*Nuc was incubated with different types of nucleic acids, including dsDNA from salmon sperm (sheared) and calf thymus (unsheared), ssDNA from calf thymus and plasmid DNA. All types of nucleic acids were completely degraded indicating that the enzyme acts in an unspecific way (Fig. 1d). Moreover, the enzyme hydrolyzed RNA (Fig. 1d). *Ec*Nuc is not affected by low concentrations of EDTA (0–10 mM), but becomes partly inactivated at higher concentrations (Fig. 1e). The nuclease displayed optimal activity at an acidic pH of 5.8 (Fig. 2b) and at a temperature of 41.7 °C (Fig. 2a). The influence of different ions and other supplements on the catalytic activity of *Ec*Nuc were evaluated, because salt and detergent tolerance is an important prerequisite for the application of nucleic acid degrading enzymes in industrial downstream processing workflows (Fig. 2c). Michaelis–Menten kinetics revealed an affinity of K_M = 202 μM and a maximal reaction velocity of v_{max} = 4559 U mg^{-1} towards sheared dsDNA (Table 1).

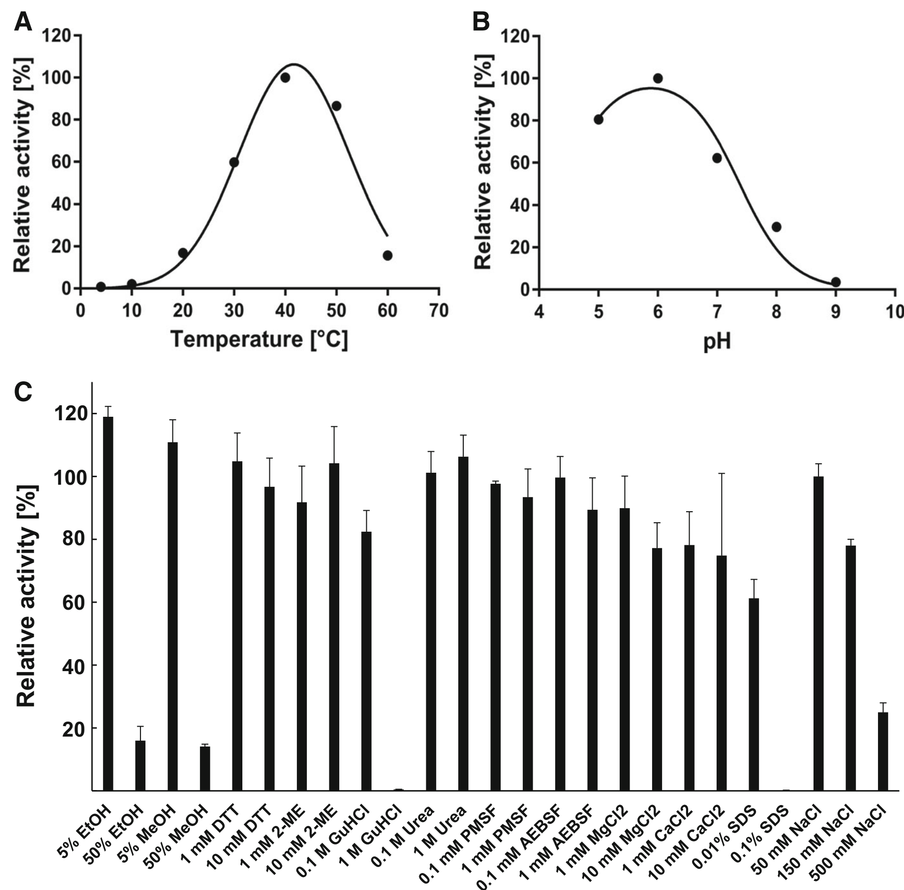

Fig. 2 Effects of temperature, pH and different chemical compounds on the catalytic performance of *Ec*Nuc. **a** An optimal temperature of 41.7 °C was determined when enzymatic activity was tested at different temperatures. **b** Variations of the pH conditions revealed an optimal pH of 5.8. Circular plasmid DNA was used as substrate. **c** Various chemical compounds were tested for activating and inhibitory effects. *Ec*Nuc was incubated for 60 min on ice in the presence of a specific compound before a quantitative activity assay at 40 °C and pH 6.0 was conducted. The enzyme is inhibited by alcohol solvents (50%(v/v) EtOH and 50% (v/v) MeOH), by chaotropic salts (1 M GuHCl) and by detergents (0.1% (w/v) SDS), respectively. Standard deviations are the result of three independent measurements. *EtOH* ethanol, *MeOH* methanol, *DTT* dithiothreitol, *2-ME* 2-mercaptoethanol, *GuHCl* guanidine hydrochloride, *PMSF* phenylmethane sulfonyl fluoride, *AEBSF* 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and *SDS* sodium dodecyl sulfate

Table 1 Kinetic properties of nucleases

	*Ec*Nuc	*S. marcescens* NucA	Bovine DNase I
K_M [μM]	202	53.8	109
k_{cat} [s^{-1}]	614	2100	176
k_{cat}/K_M [s^{-1} μM^{-1}]	3	5.4	1.6

Data of NucA and DNase I were obtained from (MacLellan and Forsberg 2001, Table 3)

Application of *Ec*Nuc to eliminate nucleic acids from crude protein extracts

Gravity flow experiments were performed to visualize the reduced viscosity of crude protein extracts that were supplemented with *Ec*Nuc (Fig. 3a). A metal-ion dependent nuclease (Benzonase) and *Ec*Nuc are both capable of degrading nucleic acids derived from lysed cells (Fig. 3b). The metal-ion dependent nuclease is not able to reduce the viscosity in crude protein extracts that were supplemented with 20 mM EDTA, while 76.0% ± 11.3% of a cell sample that was treated with *Ec*Nuc passed through the pipette tip in the gravity flow experiment, indicating the catalytic activity of the enzyme under these conditions (Fig. 3c).

Discussion

Due to their versatile application potential, non-specific nucleases have attracted considerable interest. There are several enzymes in *E. coli* that are capable to degrade nucleic acids. The nuclease domain of the bacterial toxin colicin E9 displayed catalytic activity towards dsDNA in the presence of the transition metals Co^{2+} and Ni^{2+}, while no Ca^{2+}-dependent activation of the enzyme was determined (Pommer et al. 1998). In contrast to the nuclease domain of the bacterial toxin colicin E9, activity of *Ec*Nuc is not dependent on the presence of divalent cations. Moreover, *Ec*Nuc depolymerized deoxynucleic acid, including closed circular plasmids as well as sheared and unsheared genomic DNA. It is most active at 41.7 °C and at a pH of 5.8, which is comparable to the extracellular nuclease Rv0888 from the pathogenic bacterium *Mycobacterium tuberculosis* exhibiting optimal activity at 41 °C and at a pH of 6.5 (Dang et al. 2016). Determination of kinetic parameters and comparison with commercial enzymes revealed that *Ec*Nuc exhibited higher activity than bovine DNase I, but it is less active compared with NucA from *S. marcescens* (Table 1).

During purification *Ec*Nuc and EF-Tu1 (elongation factor Tu1) coeluted from the column, indicating a putative interaction between both proteins. It has been described earlier that EF-Tu1 undergoes specific polymerization and tightly binds to DNases (Beck et al. 1978). Only a limited number of isozymes that are independent of metal ions in their catalytic region have been experimentally tested in detail and were investigated with regard towards their application potential in biotechnology. Tolerance against high salt concentrations is demanded in downstream processes, because standard buffers for protein chromatography often contain 50–500 mM NaCl. Another positive effect of high salt concentrations is that nucleic acids are enabled to dissociate from proteins at high salt concentrations. Thereby, these nucleic acids become available for degradation by salt tolerating enzymes. *Ec*Nuc is slightly inhibited in the presence of high salt concentrations. The nuclease from *S. marcescens* is not affected by concentrations of NaCl up to 200 mM (Eaves and Jeffries 1963), but becomes quickly inactivated by chelating agents. In contrast, *Ec*Nuc might be applicable in EDTA-containing cell lysis buffer, useful to increase yields of recombinant proteins due to inactivation of metal-dependent proteases. However, EDTA does not allow a metal-ion dependent affinity chromatography approach, such as Ni^{2+}-HIS-tag chromatography as a first purification step. Therefore, *Ec*Nuc in combination with EDTA-containing buffers is compatible with alternative purification approaches including hydrophobic interaction chromatography, ion exchange chromatography or the recently developed ultrahigh affinity chromatography (Vassylyeva et al. 2017).

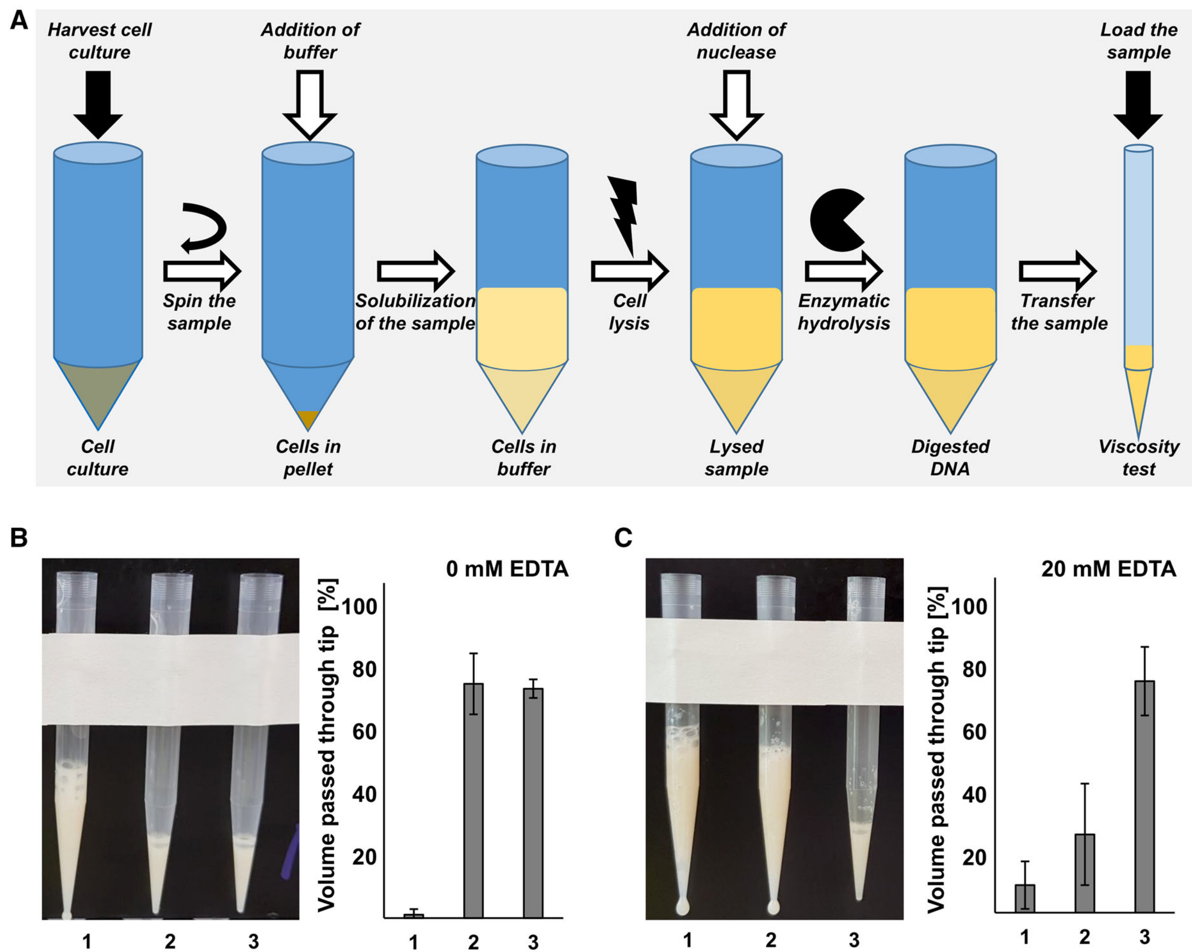

Fig. 3 Potential application of *Ec*Nuc to degrade nucleic acids in crude protein extracts. **a** Schematic illustration. **b**, **c** Documentation of gravity flow experiments to visualize the viscosity of crude cell extract samples. *1* control without enzyme, *2* metal-ion dependent nuclease (Benzonase), *3 Ec*Nuc. **b** *Ec*Nuc and a metal-ion dependent nuclease degrade the nucleic acids in the sample without EDTA and enable the crude protein extract to run through the pipette tip, while a sample containing no nuclease leads to a plugged pipette tip and the formation of a viscous drop. **c** *Ec*Nuc is also capable to reduce the viscosity of a crude protein extract sample that was supplemented with 20 mM EDTA, while the metal-ion dependent nuclease only displayed reduced activity. Bar charts aside of the pictures indicate the volume that passed through the pipette tips. Values are given as % of volume passed through the tip. The amount of cell sample that was filled into the tip at the beginning of the experiment (about 1 ml of lysed crude protein extract) was set to 100%. Standard deviations are the result of three independent measurements

Conclusions

A novel metal-ion independent nuclease from *E. coli* has been produced and characterized. Due to its substrate promiscuity, *Ec*Nuc has been shown to be applicable for the removal of different types of nucleic acids in EDTA-containing buffers for the subsequent purification of proteins from crude extracts.

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Supporting information Supplementary Figure 1—Codon usage optimized nucleotide sequence of *Ec*Nuc without predicted signal peptide. The deduced amino acid sequence is indicated below in 1-letter amino acid code.

Supplementary Table 1—Purification of recombinant *Ec*Nuc after expression in *E. coli* Veggie BL21 (DE3).

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