

# Expression of a Plant-Derived Peptide Harboring Water-Cleaning and Antimicrobial Activities

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**Abstract:** Drinking water is currently a scarce world resource, the preparation of which requires complex treatments that include clarification of suspended particles and disinfection. Seed extracts of *Moringa oleifera* Lam., a tropical tree, have been proposed as an environment-friendly alternative, due to their traditional use for the clarification of drinking water. However, the precise nature of the active components of the extract and whether they may be produced in recombinant form are unknown. Here we show that recombinant or synthetic forms of a cationic seed polypeptide mediate efficient sedimentation of suspended mineral particles and bacteria. Unexpectedly, the polypeptide was also found to possess a bactericidal activity capable of disinfecting heavily contaminated water. Furthermore, the polypeptide has been shown to efficiently kill several pathogenic bacteria, including antibiotic-resistant isolates of *Staphylococcus*, *Streptococcus*, and *Legionella* species. Thus, this polypeptide displays the unprecedented feature of combining water purification and disinfectant properties. Identification of an active principle derived from the seed extracts points to a range of potential for drinking water treatment or skin and mucosal disinfection in clinical settings. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 81: 13–20, 2003.

**Keywords:** coagulation; antimicrobial peptide; drinking water; disinfection

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## INTRODUCTION

The treatment of water to render it fit for human consumption has become a problem of central importance, both in developing and in developed countries. In developing areas, the quality of drinking water is often insufficient and hazardous to health. In developed countries, water purification processes use chemicals, despite the fact that their safety for health during long-term use and impact on the environment remain under question. As a result, it is desirable to find sustainable alternatives more friendly to human health and to the environment.

Water treatment usually comprises water clarification and disinfection. The turbidity of water often results from the presence of negatively charged particles in a colloidal structure, the clarification of which requires acceleration of the sedimentation rate. For this purpose, positively charged agents are used to neutralize the negative charges of the colloid, in a process called coagulation. In developed countries, salts of aluminum and other metals are often used (Boisvert et al., 1997; Nalm et al., 1998; van Benchosten and Edzwald, 1990), despite the concern that they may induce Alzheimer's or other disease (Crapper et al., 1973; Martyn et al., 1989; Miller et al., 1984). Although synthetic organic polymers are used increasingly for water treatment in conjunction with metal salts, efficient substitutes for the latter are not available at present (Odegaard, 1998; Parker et al., 2001).

Water disinfection also often makes use of chemical additives, with chlorine and chloramine being two of the most widely used ones. While the benefits of water disinfection are clear, concerns have also been raised over safety issues concerning disinfectants. For example, disinfectants and their by-products may be associated with increased risks of

cardiovascular diseases, cancers, and birth defects. Although such risks are low, associations with such diseases could not be ruled out unambiguously (Arbuckle et al., 2002; Bove et al., 2002; Woo et al., 2002).

Naturally occurring alternatives to currently used coagulants and disinfectants have also been considered, including ones found in cultivated plants. Of particular interest are the seeds of a tropical tree, *Moringa oleifera* Lam., as they contain an active coagulating compound traditionally used for the clarification of drinking water in rural areas of Sudan and Malawi (Eilert et al., 1981; Okuda et al., 2000). The coagulating activity of *Moringa* sp. seeds is still a matter of debate, as it has been ascribed either to low molecular mass cationic polypeptides (Tauscher, 1994) or to a non-proteinaceous compound of unknown structure (Okuda et al., 2000). In addition, such seed extracts are capable of bacterial aggregation and removal, with efficacy similar to that of aluminum salts and other commonly used water treatment agents (see Madsen et al., 1987, and references therein). These properties suggest that seed extracts may be promising alternatives to the currently used chemical water treatment agents.

In this paper we describe the expression and characterization of one of the *Moringa* sp. seed polypeptides. Our results show that it acts as a water clarification agent that coagulates particles and bacteria in suspension. In addition, we show that it also possesses an antibiotic activity that leads to growth inhibition and killing of bacteria, including antibiotic-resistant human pathogens. Thus, *Moringa* seed polypeptides might represent environment-friendly substitutes to commonly used coagulation and disinfecting agents.

## MATERIALS AND METHODS

### Plasmids

A DNA sequence was designed to encode the MO2.1 polypeptide sequence of Gassenschmidt et al. (1995) (see Fig. 1A). Recombinant or synthetic forms of this polypeptide were termed Flo in this article. The double-stranded oligonucleotide was synthesized using a PCR assembly strategy, as described previously (Horton et al., 1989). The oligonucleotide sequence was designed so that its codons are optimized for *Escherichia coli* expression and so that SapI and PstI restriction sites are located at its extremities. The pTYB11 plasmid of the IMPACT expression system (intein-mediated purification with an affinity chitin-binding tag system, New England Biolabs, Inc., Beverly, MA) was selected for cloning and expressing the *Moringa* seed Flo protein in *E. coli*. The oligonucleotide was ligated to SapI/PstI-digested pTYB11 vector so that the sequences encoding the N-terminus of the target protein Flo, an internal protein self-cleavage site (intein), and chitin-binding domain are fused. Positive clones were verified by sequencing.

### Protein Expression and Purification

Flo protein was expressed using the pTYB vectors and *E. coli* ER2566 strain (New England Biolabs). To induce ex-

pression of the fusion protein, 0.3 mM IPTG was added to an exponentially growing culture at an  $A_{600}$  of 0.5–0.6 during 2 h at 27°C. The bacterial culture, extract preparation, and purification conditions were as recommended by the manufacturer (New England Biolabs). In brief, a 1.5-liter bacteria culture volume ( $A_{600} = 0.5–0.6$ ) was centrifuged, and cells were lysed by sonication. Extracts were clarified by centrifugation and loaded onto an equilibrated chitin bead column. After being washed, the column was filled with 50 mM DTT containing buffer, which was incubated in the column for 40 h at room temperature to allow for self-cleavage of the Intein-containing fusion peptide. Flo was eluted, and its presence was confirmed by gel electrophoresis. Finally, precursor protein was eluted with stripping buffer, and the column was recycled.

Total cell protein extracts were analyzed using 10% SDS-PAGE (Laemmli, 1970). For protein quantification, gels were stained using cypro-orange and analyzed using scanning software (Storm 840, Molecular Dynamics, Sunnyvale, USA). The Flo polypeptide was analyzed by Tris-tricine SDS-PAGE (Schagger and von Jagow, 1987). For gel fixing and staining, a protocol suitable for small basic proteins was followed (Steck et al., 1980).

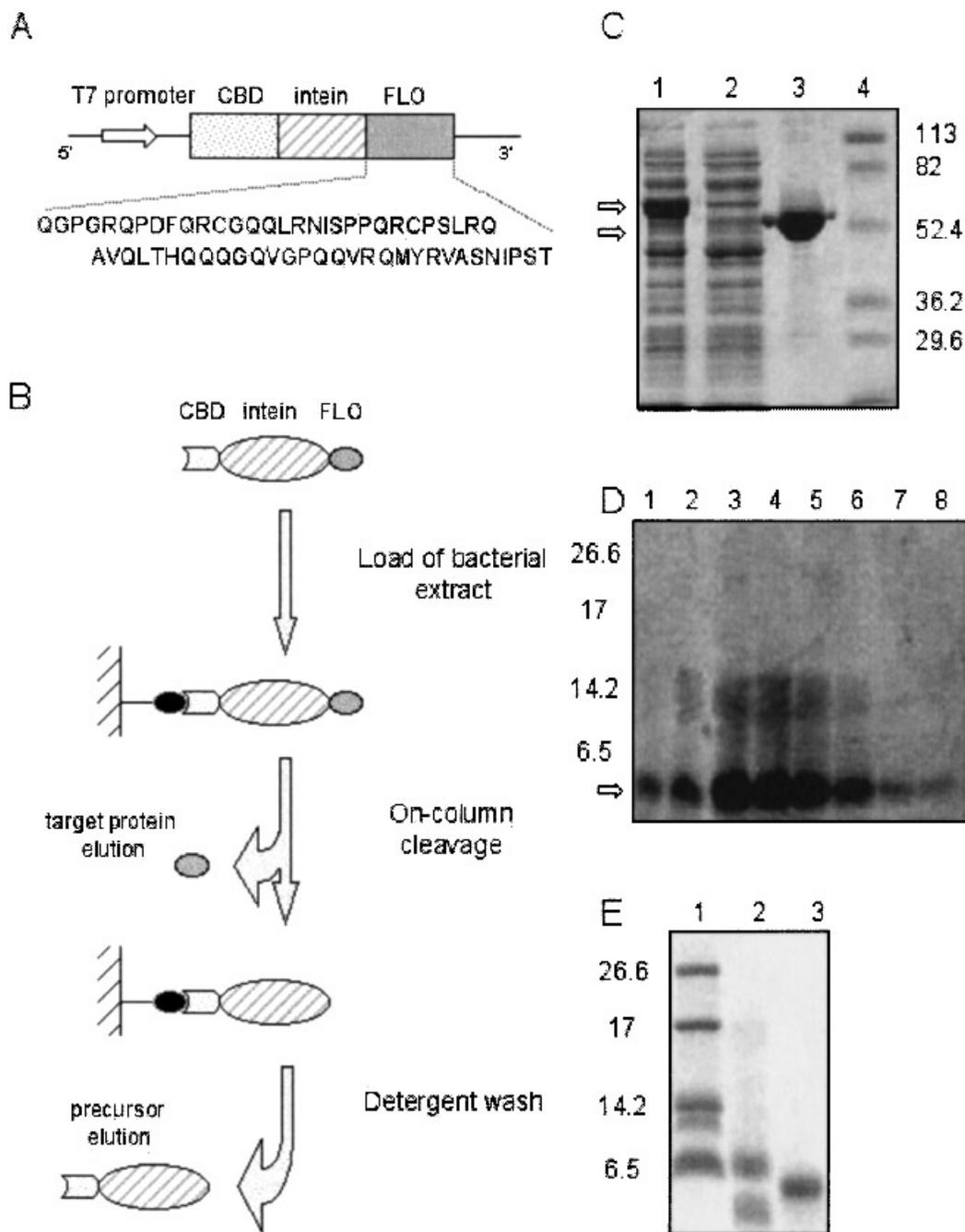
Synthetic Flo was synthesized at the Peptide-Protein Chemistry Facility of the University of Lausanne using the amino acid sequence of *Moringa oleifera* Lam. seed component M.O. 2.1 (Gassenschmidt et al., 1995). *Moringa* sp. seed extract was obtained as a commercial preparation (Phytofloc, Optima Environment Inc., Nyon, Switzerland). Briefly, a ground presscake of *Moringa* sp. seeds was mixed with saltwater at 1:5 w/v ratio. The extract was filtered and heated at 80°C. Precipitated solids were removed by centrifugation and the clarified liquor was concentrated by filtration through 5-kDa cut-off membranes. Further inquiries on the use of Phytofloc and Flo (patent pending) may be directed to Optima Environment SA.

### Coagulation Assays

Coagulation activities were evaluated using a 1 mg/mL suspension of 3.5–7  $\mu$ m diameter glass particles (Spherglass 5000, Potters-Ballotini, U.K.) in 2 mL of 50 mM phosphate buffer, pH 7.0, to mimic turbid water. Stirring was kept continuously at 800 rpm, and OD was recorded at 500 nm (LabVIEW software/National Instruments Corporation) in a spectrophotometer. After 5 min of continuous stirring, the compound to be tested was added to a final concentration of 10  $\mu$ g/mL, unless otherwise noted, and stirring was continued for 15 min. The flocculation efficiency was estimated from a linear regression performed on time points corresponding to 4 min before the addition of the flocculating preparation (basal sedimentation) and 4 min after the addition of the flocculating preparation (coagulation-mediated sedimentation).

### Antibacterial Effects

Exponentially growing *E. coli* ER2566 was centrifuged and suspended in a same volume of 10 mM phosphate, pH 7.0,



**Figure 1.** Flo expression and purification. (A) Structure of the Flo fusion protein expression vector. The Flo coding sequence (shaded box) was inserted downstream of sequences encoding the self-cleavage intein protein domain (striped box) fused to the chitin-binding domain (CBD, dotted box), under the control of a regulated T7 phage promoter. Sequence of the Flo polypeptide, as released from the intein sequence after self-cleavage, is shown below. (B) Scheme of the purification process. The bacterial extract containing the fusion protein was loaded onto a chitin-linked (closed ellipse) bead column, where the fusion protein is retained through its chitin-binding domain. The column was then incubated with thiols, which results in a specific self-cleavage of the intein, releasing the Flo polypeptide. The remainder of the fusion protein is then eluted in detergent buffer to recycle the column. (C) SDS-PAGE analysis of bacterial extracts. Equivalent fractions of the purification intermediates were loaded as follows: crude extract from IPTG induced cells (lane 1); chitin column flow through (lane 2); eluate of remaining part of the fusion protein after self-cleavage (lane 3); protein molecular weight marker (lane 4); At the left, the upper arrow indicates the fusion protein (61.5 kDa) and the lower arrow indicates the fusion protein after cleavage and elution of Flo (55 kDa). (D) Tris-tricine SDS-PAGE analysis of Flo eluate fractions. Lanes 1 and 2: 1 and 2  $\mu$ g of chemically synthesized Flo were loaded, respectively. Lanes 3–8: sequential fractions of Flo elution. At the right, the position of markers is indicated in kDa. The arrow indicates the position of Flo. Trace amount of polypeptides whose migration corresponds to multimers of Flo was occasionally noted in highly concentrated fractions (lanes 2–6). (E) Tris-tricine SDS-PAGE analysis of synthetic Flo and seed extract; 2.5  $\mu$ g of either total protein seed extract (lane 2) or synthetic Flo (lane 3) was resolved in parallel. Masses of molecular weight markers (lane 1) are as indicated on the left.

buffer. Alternatively, incoming water was collected at a waste-water treatment facility. Phytofloc, synthetic Flo, or BSA was added and incubated with bacteria as specified in the figure legends at 37°C. Bacteriostatic effects were measured by adding LB growth medium to the bacterial suspension to obtain  $A_{600} = 0.1$ , the cultures were incubated at 37°C under agitation, and the culture growth was followed by  $A_{600}$  measurements. Alternatively, particles and viable cells were counted either directly after the incubation or after additional washes of the cells in phosphate buffer to remove flocculating proteins. Assays of particle size and number were performed with a CASY cell counter (Schärfe System Inc., Reutlingen, Germany), and cell viability was assessed by plating bacterial suspensions on non-selective LB medium dishes. Standard deviations of independent measurements ranged from 2% to 20% of the mean.

### Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentrations (MBC)

Methicillin-resistant *Staphylococcus aureus* P8 (Entenza et al., 2001), *Streptococcus mitis* (Entenza et al., 1999), *Streptococcus pneumoniae* (Moreillon et al., 1990), *Enterococcus faecalis* (clinical isolate, Lausanne University Hospital), *Legionella pneumophila* serotype 1 (nosocomial isolate, Ticino Bacterioserological Institute, Lugano, Switzerland), *Streptococcus pyogenes* ATCC 19615, and *E. coli* ATCC 25922 (NCCLS strain collection) were grown at 37°C without aeration, in either Mueller Hinton broth (MHB), buffered yeast extract medium (BYE $\alpha$ ), or buffered charcoal yeast extract (BCEY $\alpha$ ; Difco Laboratories, Detroit, MI) or on Columbia agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 4% blood. Approximately  $5 \times 10^5$  CFU/mL of bacteria were suspended in phosphate buffer as above and incubated at 37°C with indicated amounts of Flo or *Moringa* sp. seed extracts. After 24 h of incubation (48 h for *L. pneumophila*), 0.01- and 0.1-mL volumes of bacterial suspension were spread on nutrient agar, and the plates were incubated for an additional 24 h (48 h for *L. pneumophila*) at 37°C before colony counts. The MIC was defined as the lowest concentration of Phytofloc or Flo inhibiting bacterial growth. The MBC was defined as the lowest drug concentration resulting in a  $\geq 99.9\%$  decrease in viable counts as compared to the original inoculum (National Committee for Clinical Laboratory Standards, 2000). Phase contrast microscopy indicated that the decrease in bacterial viability was not due to aggregation.

## RESULTS

Previous work on the coagulating activity associated with *M. oleifera* Lam. seed extracts had indicated that the activity co-purifies with low molecular weight proteins. The sequence of one of these proteins was determined and shown to be a positively charged 6-kDa polypeptide (Tauscher,

1994). However, previous attempts to express a recombinant form of this protein and to demonstrate an associated coagulation activity were not successful. In the present study, we have addressed this issue using either a synthetic form of this polypeptide or with a bacterially produced recombinant protein.

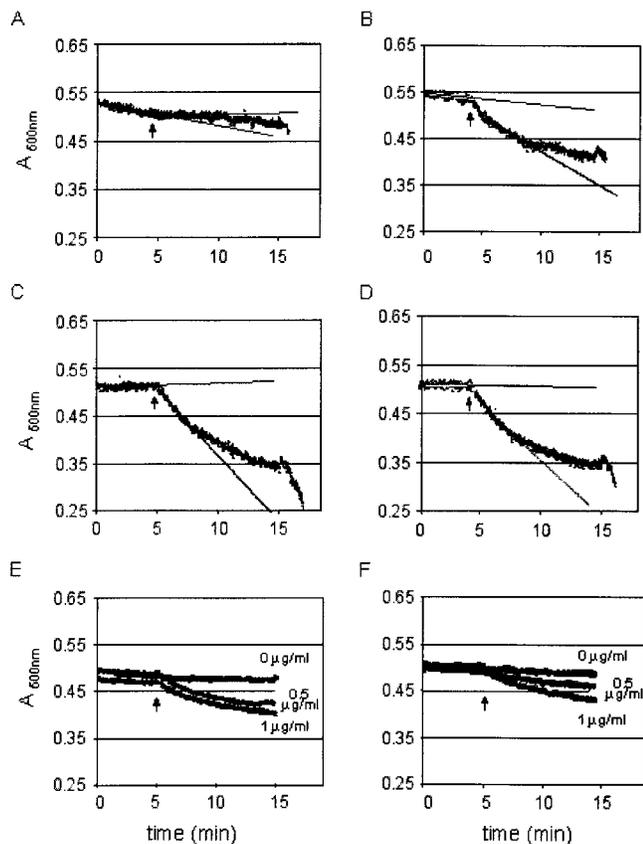
### Protein Cloning, Expression, and Purification

Using the previously determined protein sequence, we reconstructed a synthetic gene optimal for the expression in *E. coli* of the recombinant *M. oleifera* Lam. seed protein, which we termed Flo. Previous work had demonstrated that the expression of positively charged peptides may be toxic to *E. coli* (Piers et al., 1993). Therefore, the expression vector was designed such that the highly positively charged Flo is expressed as a fusion polypeptide with the negatively charged intein and chitin-binding domains (Fig. 1A). The chitin-binding domain allows for convenient purification of the fusion protein, while intein allows post-translational cleavage of the precursor protein, as described previously (Perler, 2000) (Fig. 1B). Over-expression of a fusion protein of the expected size was obtained (Fig. 1C), yielding approximately 30% of the total bacterial protein content. Purification and autocatalytic cleavage of the fusion protein allowed the recovery of approximately 1 mg of purified Flo per liter of bacterial culture (Fig. 1D).

Analysis of Flo amino acid sequence indicated significant similarity with the 8 kDa heavy chains of the napins and mabinlins, except that the N-terminal portion of the polypeptide is missing in Flo (M. Suarez and N. Mermod, unpublished results). The napins and mabinlins belong to the plant 2S albumin protein family, the most abundant storage proteins in plant seeds (Broekaert et al., 1997). Thus, Flo is expected to represent a truncated version of its *M. oleifera* Lam. seed 2S protein counterpart. As expected from this finding, bacterial or synthetic Flo migrated just below the heavy chain of the seed extract major protein (Fig. 1E).

### Coagulation Activity of Flo

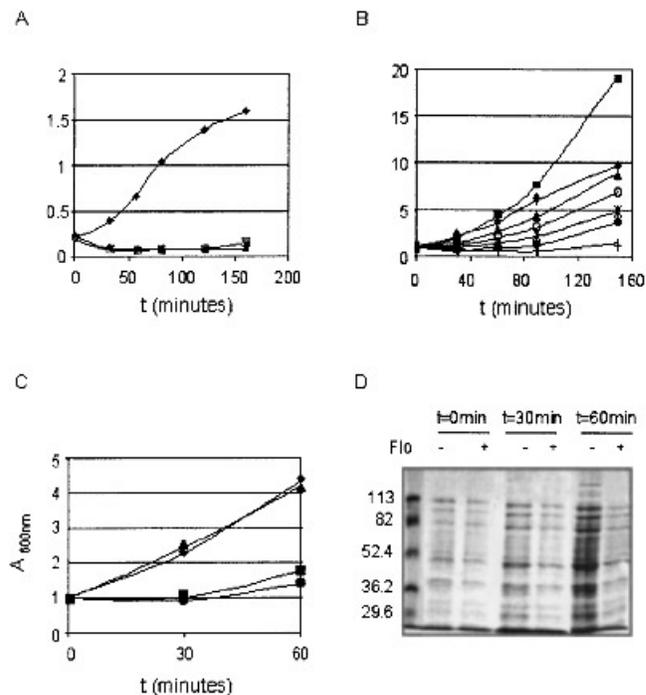
The coagulation properties of chemicals and protein extracts are usually assessed using suspensions of particles, such as glass microbeads, that mimic the negatively charged properties of particles found in natural turbid waters (Tauscher, 1994). Flo and the seed extract were therefore added to continuously agitated suspension of glass microbeads, and sedimentation was estimated by following the decrease in optical density. Little sedimentation occurred before or after the addition of buffer (Fig. 2A). However, efficient coagulation occurred with saturating amounts of seed extract (Fig. 2B) and with chemically synthesized or bacterially produced Flo (Fig. 2C,D). Use of subsaturating amounts of the synthetic peptide and of the seed extract indicated comparable activities in this semiquantitative assay (Fig. 2E,F), which correlates well with the similar amounts of the major polypeptides observed in the two preparations (Fig. 1E).



**Figure 2.** Coagulation activity of Flo. The glass particle suspension sedimentation assay was performed in spectrophotometer cells as described in Materials and Methods, and optical density measurements at 600 nm were taken at 1-s intervals. After 5 min of stirring, seed extract (B), synthetic Flo (C), or bacterially expressed and purified Flo (D), respectively, were added to a final concentration of 10  $\mu\text{g}/\text{mL}$ , as indicated by the arrow. In panel A, a similar amount of buffer only was added. The slopes of the sedimentation curves, before and after addition of the compound to be tested, were estimated by linear regression calculations as described in the Experimental protocol, and are shown as straight lines. (E, F) Titration experiments performed with the indicated subsaturating amounts of the seed extract and synthetic Flo, respectively.

### Antibacterial Effects of Flo

*Moringa* sp. seed extracts were shown previously to flocculate bacteria and to possess antimicrobial activity (Eilert et al., 1981; Madsen et al., 1987). However, the agent responsible for the flocculation activity was not identified, while the antimicrobial activity was ascribed to plant-synthesized derivatives of benzyl isothiocyanates, a known antibacterial compound. Nevertheless, we set up to evaluate the potential effects of Flo on *E. coli*. Exponentially growing bacteria were incubated with various concentrations of Flo and returned to culture conditions to monitor growth. Incubation with 2 mg/mL of either seed extract or Flo resulted in strong inhibition of bacterial growth (Fig. 3A). Growth inhibition was detectable at lower Flo concentrations, with an  $\text{IC}_{50}$  of approximately 100  $\mu\text{g}/\text{mL}$  (Fig. 3B). Incubation with bovine serum albumin, used as a negative control, indicated that the antibacterial effect is specific to Flo.



**Figure 3.** Effect of Flo on *E. coli* culture growth. (A) Effect of the *Moringa* sp. seed extract and of bacterial Flo protein. An exponential-phase *E. coli* culture was centrifuged and incubated for 2 h at 37°C in phosphate buffer alone (filled diamonds) or in phosphate buffer supplemented with seed extract (filled triangles) or with synthetic Flo (filled squares) at a final concentration of 2 mg/mL. Bacteria were then diluted to  $A_{600} = 0.1$  in LB growth medium and incubated at 37°C under agitation. Optical density measurements were then recorded as indicated at 600 nm. (B) Exponentially growing *E. coli* culture was processed as indicated for panel A except that bacteria were incubated with different concentrations of synthetic Flo (in mg/mL): 0 (filled squares), 0.1 (filled triangles), 0.25 (open circles), 0.5 (stars), 1 (filled circles), or 2 (plus signs). BSA at 2 mg/mL was used as a control (filled diamonds). (C) Assay for bacterial resistance to the antibiotic effect of Flo. *E. coli* culture consisted either of a fresh culture of bacteria (untreated bacteria) or of a culture previously incubated in presence of peptide in two successive rounds, as in panel A, where the bacteria that grew eventually were collected. These cells were incubated for a third cycle with either buffer (filled triangles) or with 2 mg/mL Flo (filled squares). Untreated cells were incubated in parallel either with buffer (0 mg/mL Flo, filled diamonds) or with Flo (2 mg/mL, filled circles). (D) Protein synthesis by bacteria incubated or not with synthetic Flo. Similar volumes of the culture of treated cells shown in panel C, either incubated with buffer (minus signs) or with 2 mg/mL of Flo (plus signs), were collected at the indicated time, bacteria were precipitated and total cell proteins were separated by SDS-PAGE and stained with Coomassie blue.

The inhibition of *E. coli* growth was found to be transitory, with resumption of growth after 3–6 h (data not shown). This suggested that some bacteria present in the culture may be spontaneously resistant to a bactericidal activity of Flo, or alternatively that the culture may eventually escape the bacteriostatic effect of Flo, for instance, after degradation of the polypeptide. In order to select for putative resistant bacteria, Flo was added to growing cells to achieve growth inhibition, and, after resumption of growth, cells were collected and incubated again with Flo. Bacteria that had grown were challenged for a third time with the polypeptide, and Flo inhibited cell growth in the same

way as observed with untreated cells (Fig. 3C). Thus, the ability of the culture to escape the effect of Flo is unlikely to be due to a minor proportion of previously resistant bacteria. To ascertain that the decrease in absorbance of the culture after the addition of Flo is due to growth inhibition rather than to the aggregation or sedimentation of the bacteria, total cell extract proteins were resolved by SDS-PAGE. This showed that bacteria treated with Flo did not synthesize proteins, in contrast to control cells (Fig. 3D). Therefore, Flo blocks *E. coli* biomass accumulation and prevents cell growth.

Visual inspection of Flo-incubated *E. coli* revealed that the peptide aggregated the bacteria, as indicated by the appearance of defined particles or flocs (data not shown). Analysis of the size and number of bacterial particles with a cell counter revealed that Flo causes an increase in the partitioning of cells in particles greater than 2  $\mu\text{m}$ , indicative of cell flocculation (Fig. 4A). Thus 6 mg/mL of Flo led to flocculation of 50% of the cells. Cell aggregation was accompanied by a decrease in the count for viable cells when spread over solid growth medium, which might conceivably result from cell death and/or from cell aggregation.

To determine if Flo has a bactericidal activity, flocs were resolved by several cycles of washing to remove Flo, and the cell size and number were determined (Fig. 4B). With up to 2 mg/mL of Flo, cells were recovered with a size profile consistent with complete resolution of the flocs. However, treatment with Flo significantly decreased the number of recovered cells, with an  $\text{IC}_{50}$  value of approximately 1 mg/mL. Flo at 6 mg/mL and above decreased particle counts to background values of 10 particles/mL and lowered viable cell count by 3 orders of magnitude, indicating that cell lysis had occurred, while approximately 99% of the remaining particles were non-viable (Fig. 4C,D). This clearly shows that Flo has a bactericidal activity.

To address whether Flo might be used to disinfect contaminated water, samples from the input of a waste-water treatment facility were tested similarly. Again, concentrations of 1–6 mg/mL of Flo were found to decrease viable cell counts by several orders of magnitude (Fig. 4D), implying a bactericidal activity on a range of bacteria and conditions.

To further assess Flo antimicrobial specificity, several Gram-positive and -negative bacteria were tested using protocols standardized for the assay of antibiotic compounds (National Committee for Clinical Laboratory Standards, 2000). A bacteriostatic effect was observed against several human pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *L. pneumophila*, and minimal inhibitory concentration (MIC) values ranged between 0.8 and 5 mg/mL of Flo between 5 and 20 mg/mL for the seed extract (Table I). Bactericidal activities (minimal bactericidal concentration, MBC) were also observed against these bacteria. Interestingly, such organisms are among the most problematic human pathogens resistant to commonly used antibiotics (Entenza et al., 2001). In contrast, Flo antibacterial activity was relatively

lower on several other bacteria, including *E. coli*. This latter result is consistent with our previous finding that moderate concentrations of Flo mediate transitory bacteriostatic effects of *E. coli* which would not be detected over the 24 h time scale of the MIC and MBC assays. Thus, Flo displays selective antibacterial effects on a range of Gram-positive and Gram-negative human pathogens.

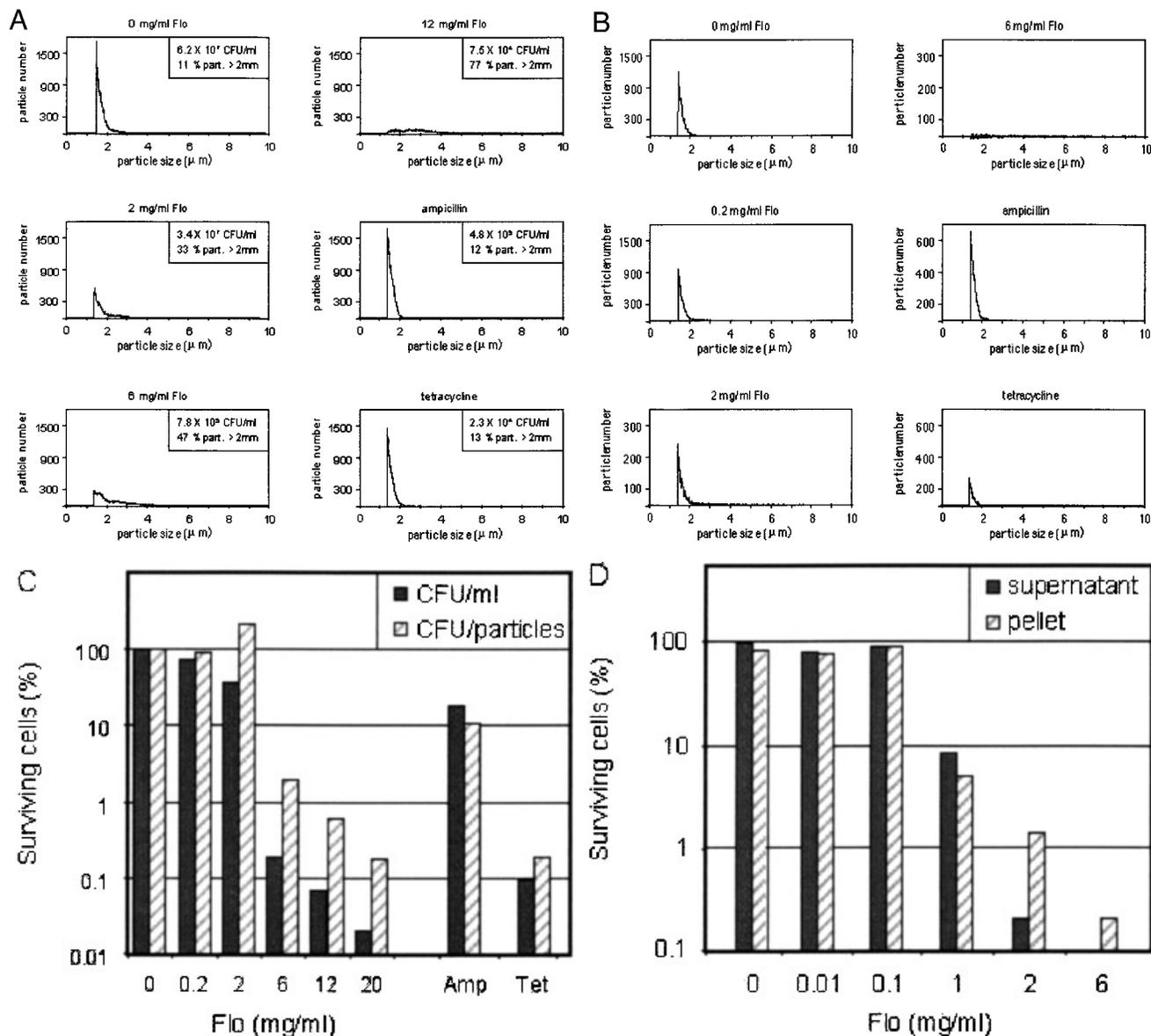
## DISCUSSION

The purpose of this study was to identify active components in *Moringa* sp. seed extracts and to determine if recombinant derivatives such as the Flo polypeptide might have a coagulation and/or flocculation activity. Our results show that high yields of Flo can be obtained from *E. coli*. The coagulation test results showed a very efficient coagulation activity of the synthetic and bacterially produced Flo polypeptide. This effect was observed using two models for water clarification: the coagulation of glass microparticles and the flocculation of bacteria. These findings indicate that the Flo polypeptide possesses hallmark characteristics of efficient water clarification.

Inspection of the Flo sequence indicated that it is extremely rich in positive charges. This is reminiscent of the so-called peptide antibiotics found in animal and plants that display a bacteriostatic or bactericidal activity (Zasloff, 2002). Furthermore, Flo displays significant homology to part of the Napins and Mabinlins 2S albumin seed proteins, some of which possess antimicrobial activities (Broekaert et al., 1997). Therefore, we tested a possible antibiotic activity of Flo and found that it displays bacteriostatic and bactericidal activities on several Gram-positive and Gram-negative bacteria. *Moringa* sp. seeds have been shown to contain one or several antibiotic principles against a wide range of Gram-positive and -negative bacteria and against fungi. This antibiotic activity was previously attributed to plant-produced benzyl isothiocyanate derivatives (Eilert et al., 1981). Our study shows that at least part of the antibiotic activity of *Moringa* sp. seed extracts may stem from Flo-like polypeptides.

Antimicrobial peptides have attracted increasing attention recently because they can efficiently kill fungi and bacteria that are otherwise resistant to many commonly used antibiotics. They act by forming channels in bacterial membranes or by inhibiting essential enzymes, leading to cell death (Zasloff, 2002). The antibacterial effect of Flo might conceivably result from similar activities or from its bacterial flocculation effect. The latter possibility is unlikely, as the bacteriostatic action of Flo is exerted at lower concentrations than its flocculation effect. For instance, Flo concentrations required to obtain half-maximal effects on *E. coli* are 0.1 mg/mL for the bacteriostatic action, 1 mg/mL for the bactericidal activity, and around 6 mg/mL for cell flocculation. Furthermore, Flo causes cell death, which is not commonly associated with cell flocculation by chemical agents.

Results presented here suggest multiple uses for Flo-like polypeptides. *Moringa* sp. seed polypeptides may be valu-



**Figure 4.** Effect of Flo on bacterial cell flocculation and viability. (A) Effect of the synthetic Flo protein on the cell particle size and number. An exponential-phase *E. coli* culture was centrifuged and incubated for 2 h at 37°C in phosphate buffer alone or in phosphate buffer supplemented by synthetic Flo at the indicated final concentration, or with 0.1 mg/mL ampicillin or 0.01 mg/mL tetracycline. Cells were then analyzed in a cell counter, and particle number is represented as a function of size. The inset shows the number of colony-forming units (CFU)/mL, scored after the suspension was spread on non-selective solid media, and the proportion of particles with a size above 2 µm, expressed as the percentage of the total number of counted particles. (B) Recovery of cells after incubation with Flo and Flo removal. Cells were incubated as described for A and then subjected to multiple wash cycles with phosphate buffer to remove Flo, as described in the Experimental protocol section. Cells were then analyzed in the cell counter as in A. (C) Effect of Flo on *E. coli* cell viability. Cell suspensions, incubated and washed as described in B, were spread on non-selective solid media, as in A. The cell viability is expressed as colony-forming unit values divided either by the volume of the original cell culture or by the total number of particles determined in B. Values are normalized to those obtained for cells incubated in phosphate buffer only (0 mg/mL), which was assigned a value of 100%, and ranged from  $1 \times 10^9$  to  $5 \times 10^9$  CFU/mL. (D) Effect of Flo on bacterial cell viability. Heavily contaminated waters from the input of a waste-water treatment facility were mixed with the indicated amount of Flo and incubated for 10 min, before spreading supernatant or sediment fractions on nonselective solid media as in A and incubating at 30°C for 24 h. Cell viability is expressed as colony-forming units, and values are normalized to those obtained for cells supplemented with phosphate buffer only (0 mg/mL), which were assigned a value of 100%. Initial bacterial loads ranged from  $1 \times 10^5$  to  $7 \times 10^5$  CFU/mL.

able alternatives to chemicals commonly used as food preservatives or for water disinfection and clarification. These polypeptides are unlikely to have toxic effects, as the seeds are currently used for the traditional treatment of drinking water and for the preparation of oil and various foods. Another advantage of treating water with such polypeptides is

their biodegradability, unlike aluminum salts, for example, which remain as contaminants of treated waters and of the sediments. Finally, Flo and *Moringa* sp. seed proteins exert selective bactericidal effects and may clear water from several water-borne human pathogens. Clearing water pipes feeding both drinking water and centralized air conditioning

**Table I.** Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of Flo on test organisms (in mg/mL).

Bacteria	Seed extract		Flo	
	MIC	MBC	MIC	MBC
<i>Staphylococcus aureus</i>	9–18	12–32	2–5	5–10
<i>Streptococcus pyogenes</i>	12	12–24	2–5	2–5
<i>Streptococcus mitis</i>	50	>50	10	10
<i>Streptococcus pneumoniae</i>	16–32	16–32	1	2.5
<i>Enterococcus faecalis</i>	>50	>50	10	>10
<i>E. coli</i>	50	>50	10	>10
<i>L. pneumophila</i>	5	5	0.8	2–3

systems is an everlasting issue in modern infrastructures. The properties of Flo, and the fact that *Moringa* seeds can be obtained and processed at a large scale (Y. Poirier, personal communication), indicate that *Moringa* sp. proteins may be a viable alternative for such applications.

The finding that recombinant Flo has antibacterial activities indicates further potential biomedical applications. Flo was shown to kill several human pathogens, such as *Staphylococcus*, *Streptococcus*, and *Legionella* species, including strains resistant to commonly used antibiotics. Decolonization of patients carrying multiresistant staphylococci and streptococci by topical application has proved critical in interfering with the spread of resistant bacteria. Selectively targeting the pathogens while sparing the commensals is key to such interventions. Because of their selectivity for several human pathogens that colonize mucosal surfaces, Flo-like polypeptides might fit such applications.

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