Development and production of recombinant human fibronectin using the transgenic silkworm system

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Abstract

Fibronectin is an important extracellular matrix protein and widely used as a scaffold for cell culture, but commercially available fibronectins are typically derived from blood, which carries the risk of pathogen contamination and batch-to-batch variability. To address these issues, we developed two novel recombinant human fibronectins: Fibronectin Neosilk®, Plasma and Fibronectin Neosilk®, Cellular. The recombinant human fibronectins were produced using transgenic silkworms that synthesized them in their middle silk glands and secreted them into the cocoons. To maintain the transgenic silkworms under the aseptic conditions, they were reared with the sterilized plant-derived artificial diet in a clean room. The secreted fibronectins in the cocoons were extracted by soaking them in a buffer solution and purified by two steps of liquid chromatography with agarose-based resins. Thus, we successfully produced high quality and safe recombinant human fibronectins using the transgenic silkworms.

Fibronectin Isoforms

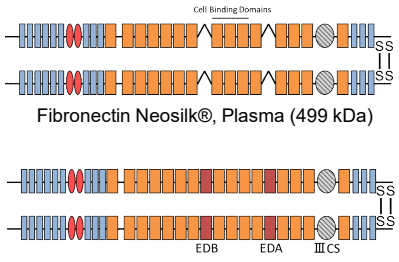


Figure 1. There exist multiple fibronectin isoforms. Plasma fibronectin is synthesized by liver hepatocytes and secreted in the blood plasma, while cellular fibronectin is synthesized by a variety of cells as a major component of extracellular matrices. A recombinant fibronectin denoted "Fibronectin Neosilk®, Plasma" is formed from subunits without extra type III repeats (EDA and EDB), while another fibronectin "Fibronectin Neosilk®, Cellular" is from subunits with EDA and EDB. Both fibronectins were synthesized as homo-dimers of the subunits by a pair of disulfide bonds at the C-terminal.

Generation of Transgenic Silkworms Expressing Human Fibronectins

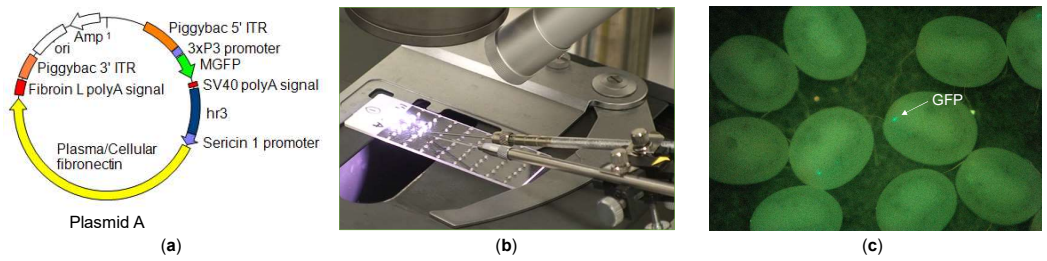


Figure 2. Genetic recombination was performed using the *piggyBac* transposon system. Injection of two plasmids (A and B) into silkworm eggs (**a, b**): Plasmid A carried the expression cassettes of the fibronectin and GFP marker, and plasmid B encoded the *piggyBac* transposase (Tamura *et al.* (2000)). The *piggyBac* transposase is expressed in the eggs, leading to the integration of the expression cassettes into the silkworm genome. When the integration occurs in germ cells, the cassettes are inherited by the next generation. We can detect the integration by observing the fluorescence from the GFP marker gene (**c**).

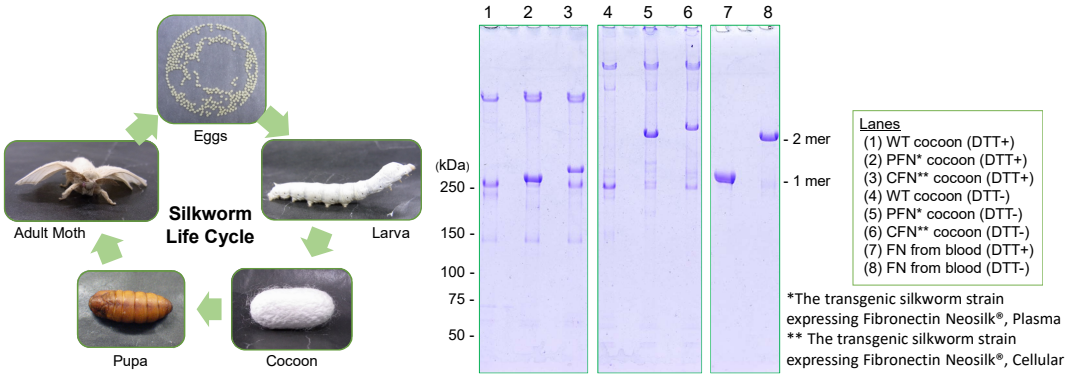
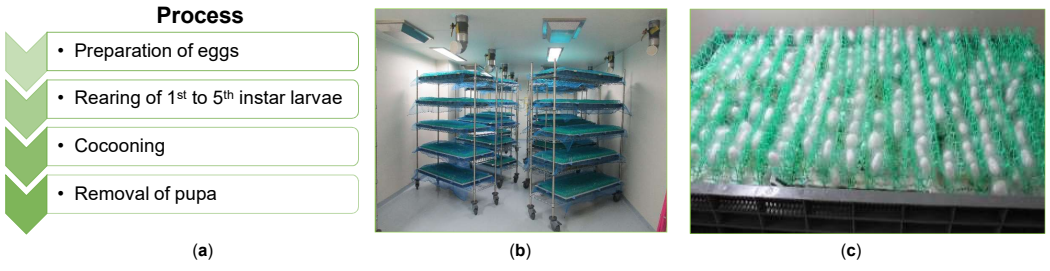


Figure 3. After hatching from eggs, silkworm larvae (0.3–0.4 mg) grow rapidly up to 1.3–1.6 g in 20 days. Then, the larvae start spinning silk threads, making cocoons of 60–80 mg (**a**). In the generated transgenic silkworms, the recombinant human fibronectins were synthesized in the silk middle glands and secreted into the cocoons. The fibronectins contained in the cocoons were extracted by socking the cocoons in a buffer solution (20 mM phosphate (pH 7.0), 4 M urea, 0.1% Triton X-100) for one hour. SDS-PAGE analysis of the extracts revealed the presence of the fibronectin bands corresponding to a monomers and dimers under reducing (DTT+) and non-reducing conditions (DTT−), respectively, as in the case of the native fibronectin from blood plasma (**b**).

Large-Scale Silkworm Rearing and Cocoon Production



Silkworm strain	Reared silkworm	Cocoon yield	Average weight of a cocoon
PFN	18,000	707 g	64.3±3.7 mg
CFN	18,000	640 g	65.3±7.1 mg

(**d**)

Figure 4. As a silkworm moth lays 200–300 eggs, the production scale can be increased exponentially by increasing the number of the silkworm moths. The silkworm rearing under aseptic conditions is realized by rearing larvae in a clean room with a sterilized plant-derived artificial diet (**a**–**c**). We started the breeding with 18,000 hatched larvae, and finally obtained 640–700 g of cocoons containing the recombinant fibronectins (**d**).

Optimization of the Extraction Condition

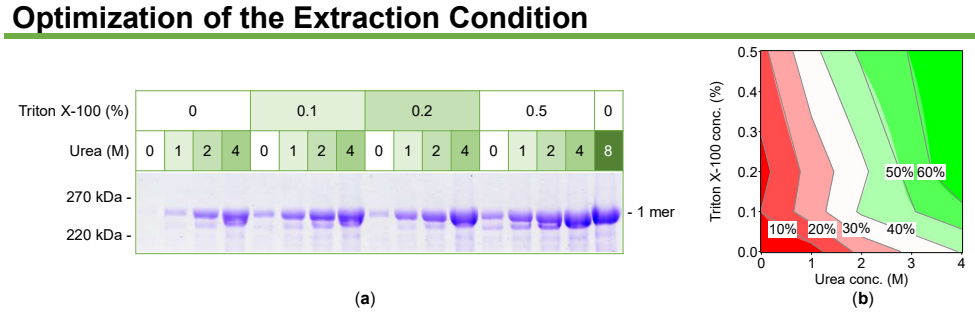
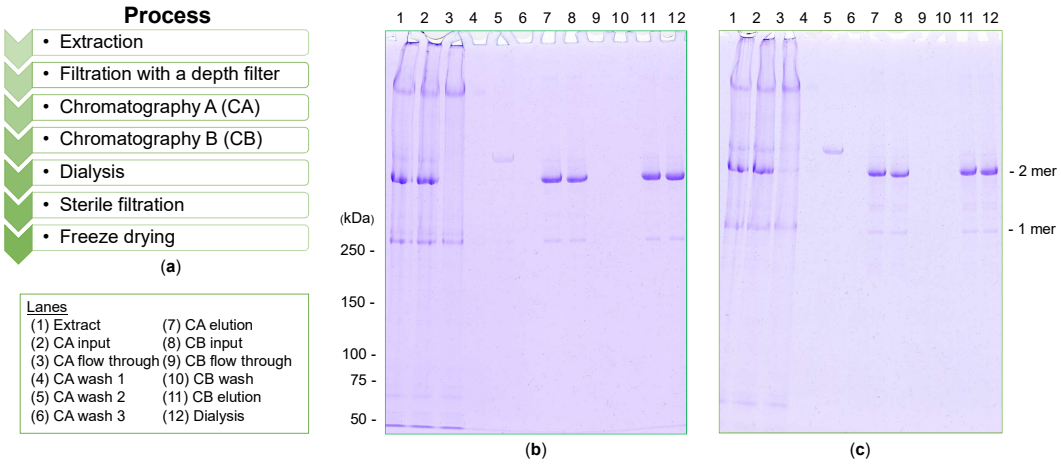


Figure 5. The extraction rate of the fibronectin from the cocoon was increased by adding Triton X-100 and urea to the extraction buffer. The effect of those additives was analyzed the extracts prepared using the buffers with different concentrations of Triton X-100 (0–0.5%) and urea (0–4 M). The extraction rate was calculated based on the ratio of the band intensities on the SDS-PAGE gel (DTT+) of the extracted fibronectin in various extraction buffers to those of the totally extracted fibronectin using the buffer containing 8 M urea (**a**). Increasing urea concentration efficiently enhanced the extraction rate, and the addition of Triton X-100 further improved the extraction rate (**b**).

Purification

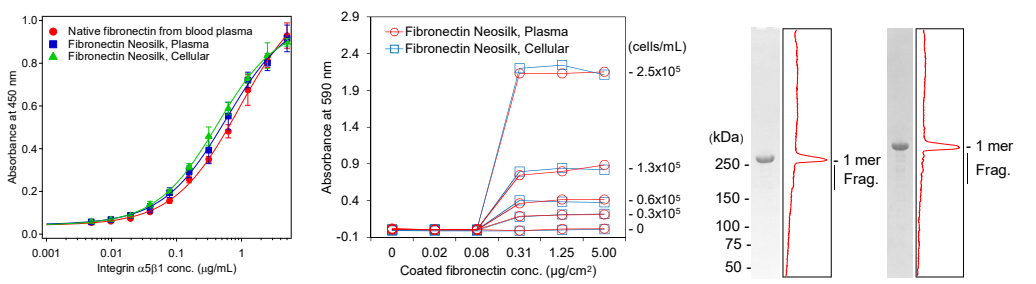


Product	Cocoon used for extraction	Purified fibronectin	Purified fibronectin per a cocoon
Fibronectin Neosilk®, Plasma	15 g	125 mg	535 μg
Fibronectin Neosilk®, Cellular	20 g	122 mg	398 μg

(**d**)

Figure 6. The recombinant fibronectins were extracted and purified from the cocoons (**a**). Aliquot of each process solution was sampled and analyzed by SDS-PAGE (DTT−) for Fibronectin Neosilk®, Plasma (**b**) and Cellular (**c**). Impurities (i.e. cocoon proteins) in the extract were efficiently removed by two steps of chromatography using agarose-based resins, Capto adhere and Capto MMC (Cytiva). Dialysis and freeze drying processes did not cause aggregation and fragmentation of fibronectins. These purification processes from 15 g and 20 g of the cocoons yielded 125 mg and 122 mg of Fibronectin Neosilk®, Plasma and Cellular, respectively (**d**).

Characterization



Property	Method	Fibronectin Neosilk®, Plasma	Fibronectin Neosilk®, Cellular
Purity	SDS-PAGE (DTT+)	93%	89%
Endotoxin	Colorimetric method	< 2 EU/mg	< 2 EU/mg
Mycoplasma	NAT method	Negative	Negative
Sterility	Direct method	Negative	Negative

(**e**)

Figure 7. Fibronectins reconstituted from lyophilized powder (Fibronectin Neosilk®, Plasma and Cellular) were characterized. The binding assay of the immobilized fibronectins to an integrin α5β1 revealed that Fibronectin Neosilk®, Plasma and Cellular have similar binding affinities to each other, and have similar to or slightly stronger affinities than the native fibronectin from blood plasma (**a**). Human fibroblasts were attached to the fibronectins when coated to dished at the concentrations of more than 0.31 μg/cm^2 (**b**). Purities of the fibronectins were analyzed by SDS-PAGE (DTT+) (c, d), revealing that 7–11% of the product-related impurities, fragmented fibronectins, were detected from the purified fibronectins. Contaminations of endotoxin, mycoplasma, and microorganisms were under the detection limits in both the purified fibronectins (**e**).

Conclusion

- We generated the transgenic silkworms synthesizing the recombinant human fibronectins in the middle silk glands and secreting them in the cocoons.
- The cocoons containing the fibronectins were produced by rearing the silkworms under aseptic conditions in the clean room with sterilized plant-derived artificial diet.
- The highly purified recombinant human fibronectins were obtained by two-steps of liquid chromatography using agarose-based resins.
- The integrin binding affinities of the recombinant fibronectins were similar to or slightly stronger than native fibronectin derived from blood plasma. No contaminations were observed for endotoxin, mycoplasma and microorganisms.
- The silkworm rearing and purification processes were rationally designed to meet the specifications required for the products used as a scaffold for cell culture, which will promise a stable supply of the high quality and safe recombinant human fibronectins.

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