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Research Article

Expression, purification and functional validation of a cancer-associated isoform of the HBx protein from human hepatitis B virus

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A B S T R A C T

The human hepatitis B virus (HBV) causes hepatitis B, a liver infection that can be acute or chronic. HBV encodes four proteins, among which the X protein (HBx) plays a critical role in viral replication. During chronic HBV infection, in which the viral DNA is integrated into the host genome, the $HBx_{1\text{-}120}$ isoform, comprising the N-terminal 120 residues, is highly expressed. Here, we describe a protocol for the recombinant overexpression and purification of untagged $HBx_{1\text{-}120}$ from bacterial cells. The procedure is compatible with stable isotope labelling in minimal media. Following cell lysis, $HBx_{1\text{-}120}$ was recovered from inclusion bodies (IBs), solubilized in urea, and purified by ion-exchange (IEX) and size-exclusion chromatography (SEC). The purified protein was extensively characterized, including by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Functionality was confirmed by a pulldown assay with a known interacting partner, Spindlin1. This protocol provides a robust framework to obtain untagged $HBx_{1\text{-}120}$ for structural and functional *in vitro* studies.

1. Introduction

HBV infection is globally prevalent and is estimated to have affected more than two billion people during their lifetime [1]. Acute infection in many cases does not cause any symptoms, and the virus is often cleared without treatment. Conversely, a chronic HBV infection leads to serious liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). The World Health Organization (WHO) estimated in 2022 that 254 million people were chronically infected with hepatitis B and that the infection was responsible for 1.1 million deaths per year [1]. Antiviral treatments are available to mitigate liver damage, but chronic HBV infection is not yet curable.

The HBV genome is composed of partially double-stranded DNA and encodes for the surface proteins, core proteins, a polymerase and the X protein (HBx) [2]. Full-length HBx is 154 amino acids in length with a molecular weight of 16.6 kDa (Fig. 1A). Its name originates from the lack of sequence homology with other viral proteins [3]. Inside infected hepatocytes, the viral DNA is organized as covalently closed circular DNA (cccDNA) that adopts a chromatin-like state. HBx has been shown to promote cccDNA transcription and thus benefit viral replication [4]. Notably, in chronically infected HBV patients, who develop HCC, high expression of a truncated HBx isoform has been detected [5]. This isoform can derive from the integration of linear HBV DNA copies into the host genome at the site of double-strand breaks [6]. Shortened RNA transcripts arise from these integrates, which produce HBx protein that is truncated at the C-terminus. One frequent short isoform is $HBx_{1\text{-}120}$ with a molecular weight of 12.85 kDa (Fig. 1A) [6]. Due to its co-occurrence with HCC, $HBx_{1\text{-}120}$ represents a crucial piece in solving the mechanism of carcinogenicity of HBV during chronic infection.

While there is strong evidence that HBx plays a role in the development of HCC, it has been very challenging to characterize the HBx protein *in vitro*, as recombinant HBx protein is highly aggregation prone [7]. To improve solubility, cysteine-free or truncated HBx have been recombinantly produced in bacteria, without solving the need of urea to unfold the protein in IBs [8]. Alternatively, large tags, such as maltose-binding protein [9], N-utilization substance protein A or disulfide bond isomerase C have been utilized [10,11]. However, due to their larger size compared to HBx itself, those tags likely influence the binding with smaller proteins [12]. Indeed, it has been reported that HBx interacts with more than 250 proteins in the cellular environment [13]. One of them is Spindlin1, a transcriptional co-activator and reader of post-translational modifications on histone tails [14,15]. So far, putative HBx interactions remain poorly characterized at the molecular level as it has been difficult to maintain full-length HBx protein in aqueous solution for *in vitro* assays. Due to this limitation previous interaction studies have been limited to short HBx-derived peptides

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spanning known interaction domains or HBx-fusion proteins [14,16,17].

Here, we present a protocol to obtain untagged $HBx_{1\text{-}120}$ from *Escherichia coli* (*E. coli*) by isolating it from IBs and then solubilizing it in urea, followed by stepwise dialysis to obtain native-like buffer conditions. We characterized the obtained protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), chromatography profiles, UV/Vis and mass spectrometry. Moreover, using a pulldown assay, we show that the recombinant protein interacts with Spindlin1 protein in a functional assay. Isotope labelling in minimal medium allowed to record NMR spectra of amide groups in the protein backbone. Thus, we provide a method for obtaining untagged, soluble $HBx_{1\text{-}120}$ for biochemical and structural studies.

2. Materials and methods

2.1. Materials and reagents

All chemicals were purchased from Sigma/Merck, Carl Roth, SERVA, Germany. For SDS-PAGE, 4–20 % Mini-PROTEAN TGX Precast Gels from Bio-Rad (Hercules, USA) were used. Chemically competent *E. coli* BL21 (DE3) cells were obtained from the Protein Expression and Purification Platform at Helmholtz Munich, courtesy of Dr. Arie Geerlof.

2.2. Cloning and expression of $HBx_{1\text{-}120}$

The used HBx sequence belongs to the Hepatitis B virus genotype D subtype ayw (Uniprot: P03165.

Natural variant R26C - Switzerland/Strubin/1999) (Fig. 1A). Cloning reagents were purchased from New England Biolabs (Frankfurt, Germany). The pET-28a (+) expression vector was purchased from Novagen (Sigma/Merck) and HBx was inserted between NcoI and HindIII restriction sites, removing the presence of the histidine tag. Specifically, the $HBx_{1\text{-}120}$ sequence was obtained from the full-length HBx sequence as a template by amplification and restriction cloning of the first 120 amino acids into the new pET-28a (+) vector (Fig. 1B).

The vector was transformed into chemically competent *E. coli* BL21 (DE3) cells (obtained from the Protein Expression and Purification Platform at Helmholtz Munich, courtesy of Dr. Arie Geerlof), which was then grown in LB medium at 37 °C with continuous shaking at 180 rpm. Once the OD_{600} reached 0.6, IPTG was added at 1 mM to induce expression at 37°C for 4 h. For ^{15}N-labelled $HBx_{1\text{-}120}$, cells were first grown in LB medium at 37 °C. After 6–7 h, they were harvested and transferred to M9 medium containing $^{15}NH_4Cl$ (Merck, Darmstadt Germany) as the only nitrogen source (0.5 g/L). Induction and expression were identical to those in LB medium.

2.3. Isolation and solubilization of IBs

Bacteria were harvested by centrifugation at 6000×*g* at 4°C for 30 min. When necessary, centrifuged pellets were frozen in liquid nitrogen and stored at −80 °C. The pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA) with addition of 1 mg/mL lysozyme, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and DNase I (2000 Units) and sonicated using a Bandelin (Berlin, Germany) Sonopuls HD 2070.2 with a KE76 Sonotrode at 4 °C for 20 min (70 % amp, 5 s on/10 s off) (Fig. 2). Sonication was followed by centrifugation at 30,000×*g* at 4 °C for 30 min to separate the soluble supernatant from the IBs containing $HBx_{1\text{-}120}$ protein. The IBs were washed with lysis buffer with 0.1 % (v/v) Triton X-100 for 30 min and collected by centrifugation at 30,000×*g* at 4 °C for 30 min. The washed IBs were finally solubilized in solubilization buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 8 M urea, 5 mM imidazole and 5 mM DTT) at 4 °C for 48 h on a rotating mixer.

2.4. Purification from solubilized IBs

The protein was dialyzed into the cation exchange running buffer (50 mM MES, pH 6.0, 6 M urea and 5 mM DTT). The sample was loaded on a 5 ml HiTrap SP HP cation exchange column (Cytiva, Marlborough, Massachusetts, USA) and eluted with a gradient using the running buffer with 1 M NaCl. The fractions containing $HBx_{1\text{-}120}$ were pooled and concentrated using a 3K MWCO concentrator (Amicon, Merck). The concentrated sample was then loaded on a HiLoad 16/600 Superdex 200 pg size exclusion column in the SEC buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 6 M urea and 5 mM DTT), and the peak fractions were pooled. The protein was kept at 4°C for up to two weeks or flash frozen in liquid nitrogen and stored at −80 °C.

2.5. Urea removal by two-step dialysis

The urea concentration in the SEC buffer (6 M) was reduced via a two-step dialysis: First, the SEC buffer was replaced by the first dialysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 4 M urea, 1 M L-arginine and 5 mM DTT at 4°C for at least 8 h. Next, the first dialysis buffer was replaced with the second dialysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 M urea, 1 M L-arginine and 5 mM DTT) at 4°C for at least 8 h.

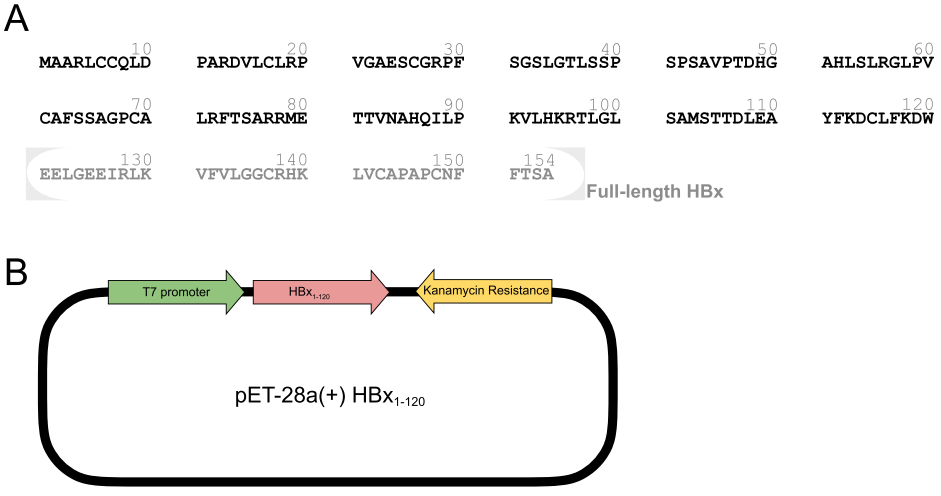

Fig. 1. A. Amino acid sequences of $HBx_{1\text{-}120}$ (black) and full-length HBx (gray). **B.** pET-28a (+) vector containing the $HBx_{1\text{-}120}$ sequence after a T7 promoter with a kanamycin resistance marker.

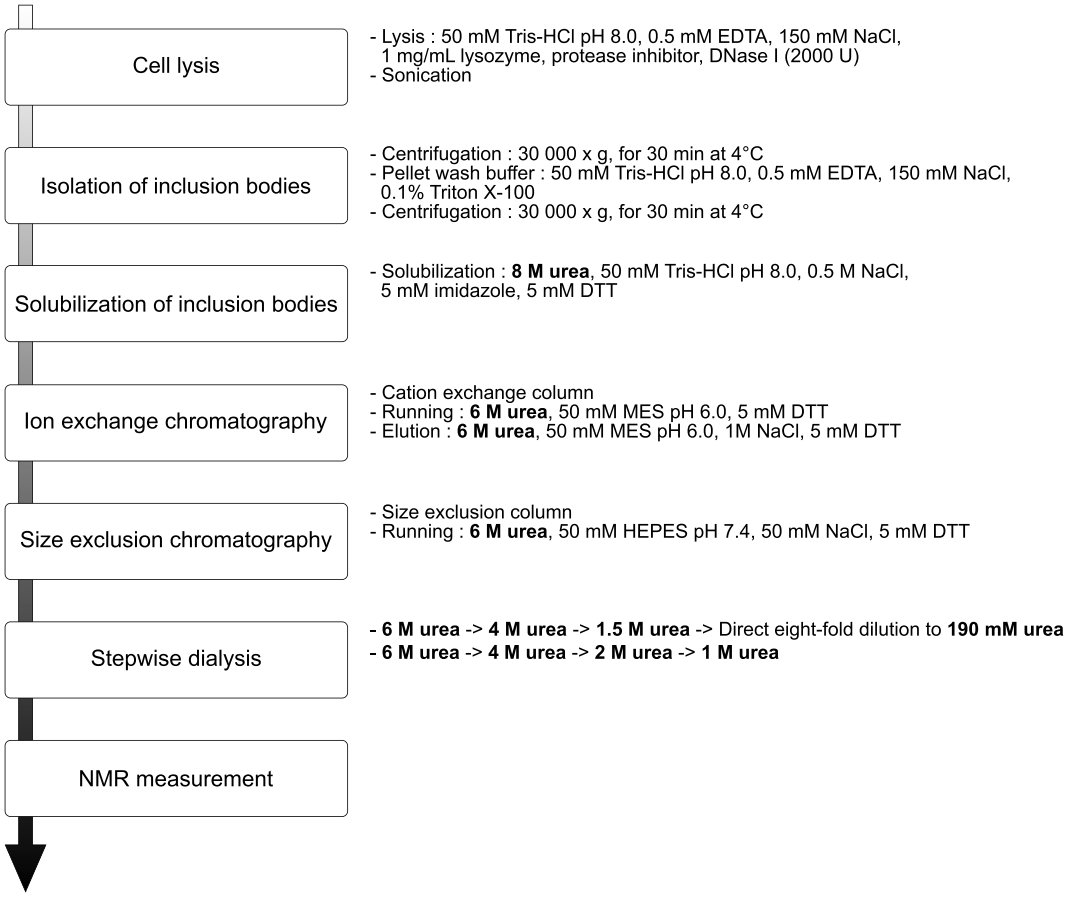

Fig. 2. Flowchart of the purification protocol for $HBx_{1\text{-}120}$ from IBs, summarizing every step along with key reagents and refolding from urea for subsequent structural and functional studies.

2.6. Electrospray ionization mass spectroscopy (ESI-MS)

Purified $HBx_{1\text{-}120}$ after the second dialysis step was concentrated to 400 μM prior to dilution to 1 μM with dilution buffer (50 mM HEPES pH 7.4 and 150 mM NaCl) to lower the urea concentration. For top-down measurements, the protein was desalted on a ZipTip with C4 resin (Merck-Millipore, ZTC04S096). The resin with loaded protein was washed thrice with a buffer containing 1 % (v/v) formic acid and $HBx_{1\text{-}120}$ was eluted with a buffer (300 μL) containing 50 % (v/v) acetonitrile and 0.5 % (v/v) formic acid.

For the ESI-MS measurements, an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA) was used via direct injection with a HESI-Spray source and FAIMS interface in a positive intact protein mode. The FAIMS compensation voltage (CV) was searched by a continuous scan. The most intense signal was obtained at −27 CV. The MS spectra were acquired with 120,000 FWHM, AGC target 100 and 2 microscans. Spectra were deconvoluted in Freestyle using the Xtract Deconvolution algorithm (Thermo Fisher Scientific, Waltham, USA).

2.7. NMR sample preparation

Two conditions were compatible with NMR spectroscopy of $HBx_{1\text{-}120}$, depending on the protein concentration required for the NMR experiments.

2.7.1. High protein concentration >100 μM

The first NMR condition was prepared in a three-step-dialysis: the first dialysis step was performed from the size exclusion buffer into a buffer containing 4 M urea, 150 mM NaCl, 50 mM HEPES pH 7.4, 0.5 M L-arginine, 5 mM DTT. The second dialysis step reached a buffer containing 2 M urea, 150 mM NaCl, 50 mM HEPES pH 7.4, 0.25 M L-arginine, 5 mM DTT. The third step brought the sample into the final buffer: 1 M urea, 150 mM NaCl, 50 mM HEPES pH 7.4, 0.125 M L-arginine, 5 mM DTT. In this final buffer the protein was concentrated to 280 μM prior to addition of 10 % D2O for the lock at the NMR spectrometer (Bruker, Billerica, USA).

2.7.2. Low protein concentration <100 μM

The protein for the second sample was dialyzed as described in section 2.5. Once in the buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 M urea, 1 M L-arginine and 5 mM DTT), the protein was concentrated to 400 μM before rapidly being diluted eightfold with the dilution buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) to a final condition that is more native-like with lower the urea and L-arginine concentration (50 mM HEPES pH 7.4, 150 mM NaCl, 0.19 M urea, 125 mM L-arginine, 0.6 mM DTT and 10 % D_2O). The final protein concentration was 50 μM. The NMR spectra were recorded in a 5 mm NMR tube (Deutero, Kastellaun, Germany) on a 800 MHz Avance III Bruker spectrometer equipped with a TCI cryogenic probe at 4 °C. SOFAST-HMQC ^{1}H-^{15}N correlation spectra were recorded [18].

2.8. Cloning, expression and purification of Spindlin1

The pET-28a (+) Spindlin1 expression vector was purchased from Genscript (Piscataway, USA). The vector contains the coding sequence covering residues 50–262 [14] with an N-terminal hexahistidine (His)-tag and a TEV (Tobacco Etch Virus protease) cleavage site, which was not cleaved in this study. The vector was transformed into *E. coli* BL21 (DE3) cells, and cells were grown in LB medium at 37 °C with

continuous shaking at 180 rpm. Once the OD_{600} reached 0.6, IPTG was added at 1 mM to induce the expression at 20°C for 16 h. Cells were then harvested by centrifugation at 6000×*g* at 4 °C for 30 min.

The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA) with addition of 1 mg/mL lysozyme, EDTA-free Protease Inhibitor Cocktail (Roche), Dnase I (2000 Units) and sonicated using Bandelin Sonopuls HD 2070,2 with a KE76 Sonotrode at 4 °C for 30 min (70 % amp, 5 s on and 55 s off). The sonicated sample was then centrifuged at 30,000×*g* at 4°C for 30 min. The supernatant was collected, applied to a HisTrap HP column (Cytiva) using buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole) and eluted with buffer B (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 400 mM imidazole).

The eluted protein was dialyzed into size exclusion buffer (50 mM HEPES, pH 7.4, and 150 mM NaCl) overnight and loaded on a size exclusion HiLoad 16/600 Superdex 200 pg column. The peak fractions were pooled and used for the pulldown assay detailed below.

2.9. Pulldown assay with Spindlin1 protein

For the pulldown assay with his-tagged human Spindlin1 protein (Uniprot: Q9Y657), the $HBx_{1\text{-}120}$ after the second dialysis at a concentration of 400 µM was diluted eightfold into a sample containing Spindlin1 in dilution buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.19 M urea, 125 mM L-arginine and 0.6 mM DTT). The final sample thus contained an equimolar mix of $HBx_{1\text{-}120}$ and Spindlin1 each at 50 µM. Subsequently, the sample was applied to a gravity flow column packed with 200 µL Ni-NTA agarose resin (SERVA, Heidelberg, Germany). The column had been pre-equilibrated with 10 column volumes buffer A. The column was washed with 10 column volumes of buffer A before finally the complex between $HBx_{1\text{-}120}$ and Spindlin1 was eluted with one column volume of buffer B.

2.10. Western blot for $HBx_{1\text{-}120}$

The protein samples from each fraction (Fig. 2) were mixed with 6x SDS-PAGE loading buffer to obtain a final concentration of 2.5 % SDS and boiled for 5 min before applying to the SDS-PAGE gel (gradient 4–20 % for gel in Fig. 4A – 18 % gels otherwise). After the run, proteins were transferred onto 0.45 µm nitrocellulose membranes (Merck, Darmstadt Germany) using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, USA) in transfer buffer (336 mM TRIS pH 9, 260 mM glycine, 140 mM tricin, 2.5 mM EDTA). The membrane was then incubated in blocking buffer, consisting of PBS-T (PBS, pH 7.4, 0.1 % (v/v) Tween-100) with 5 % dry milk at room temperature for 1 h. The membrane was incubated with the anti-HBx antibody X36C conjugated to Alexa Fluor 488 (Santa Cruz Biotechnology, Dallas, USA) diluted 1:1000 in blocking buffer at 4 °C overnight. Following antibody incubation, the membrane was washed three times with PBS-T buffer and images were visualized with iBright imaging system (Thermo Fisher Scientific, Waltham, USA).

3. Results

3.1. Purification of $HBx_{1\text{-}120}$ from IBs

The isolation of untagged HBx protein in large quantities required for *in vitro* studies has been a major challenge as the solubility of HBx is low. Here, we present a reproducible workflow to obtain $HBx_{1\text{-}120}$ from IBs after urea solubilization (Fig. 2) and to process it further for NMR measurements and functional studies.

After 4 h of overexpression at 37 °C, the cells were lysed and $HBx_{1\text{-}120}$ was found fully in IBs (Fig. 3A). This observation has been made consistently at any expression temperature, expression duration or IPTG concentration for induction. We selected a short expression time to ensure less degradation of the protein inside the bacteria before lysis (data not shown). After solubilization (8 M urea, 48 h), $HBx_{1\text{-}120}$ protein can be recovered from the IBs at considerable enrichment (Fig. 3A). Once loaded on the cation exchange column, $HBx_{1\text{-}120}$ eluted with a 20 %–30 % elution buffer gradient, which corresponds to approximately 200–300 mM NaCl (Fig. 4A). The peak fractions were pooled and loaded onto a size exclusion column (Fig. 4B). $HBx_{1\text{-}120}$ elutes at a volume which corresponds to a protein 44.3 kDa, based on a calibration performed using globular reference proteins (Fig. 4B).

The presence of urea in the elution buffer explains the altered elution behavior that is not in accordance with the true molecular size. Urea, as a denaturing agent, disrupts the native protein structure, leading to an extended unfolded conformation that increases the hydrodynamic

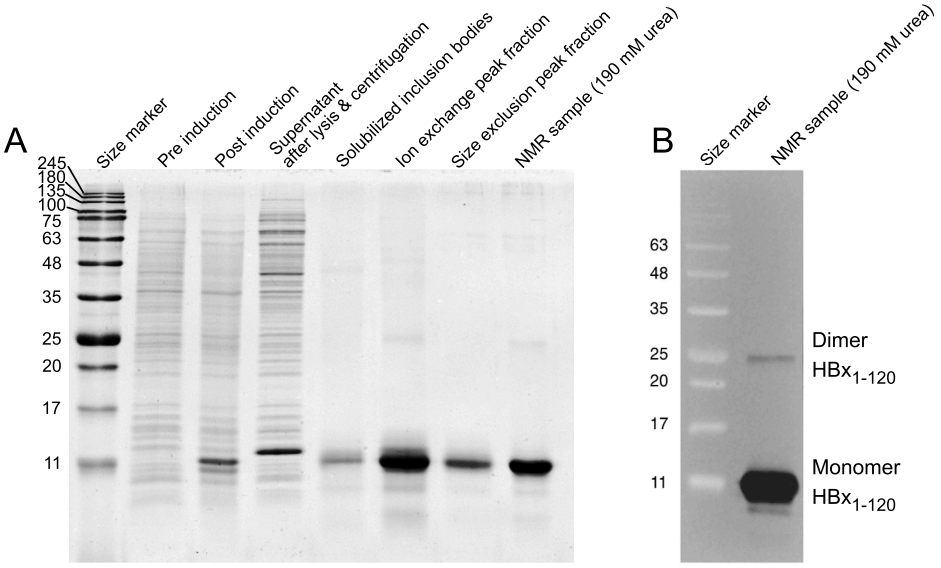

Fig. 3. A. SDS-PAGE (18 %) gel showing the step-by step purification of $HBx_{1\text{-}120}$ protein (molecular weight 12.85 kDa). The band around 14 kDa in the supernatant corresponds to lysozyme added to the lysis buffer. The band visible around 25 kDa in the lane with the final NMR sample likely derives from a dimeric form of HBx [19] and is frequently observed across independent purifications. **B.** To confirm the identity of the bands assigned to $HBx_{1\text{-}120}$, the protein was transferred onto a Western blot membrane, where the HBx-specific antibody X36C detected both the monomeric and the dimeric $HBx_{1\text{-}120}$ bands.

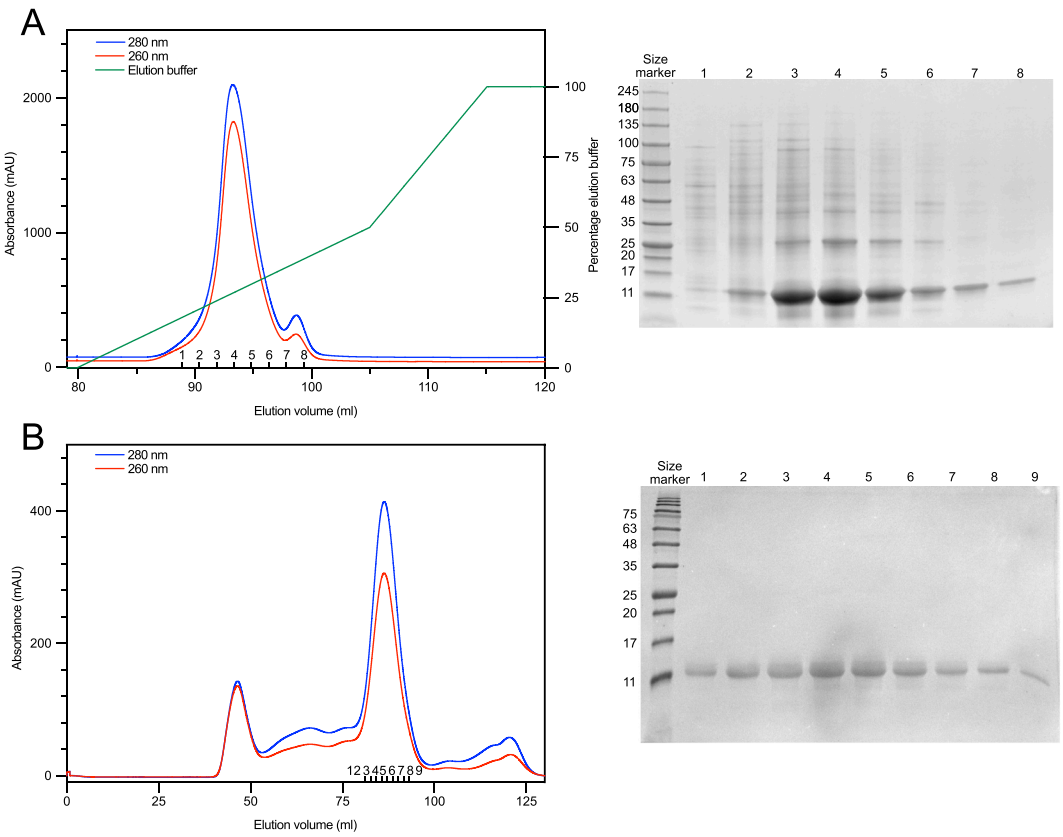

Fig. 4. A. Chromatogram of the cation exchange purification. The indicated fractions 1–8 were loaded on the SDS-PAGE (gradient 4–20 %) gel shown to the right, highlighting the enrichment of $HBx_{1\text{-}120}$ protein. The peak fractions are collected with 25 % percent of elution buffer. **B.** Chromatogram of the size exclusion purification. Fractions indicated 1–9 were loaded on an SDS-PAGE (18 %) gel and document the high purity of the final protein sample. The peak around 45 ml corresponds to the column void volume.

volume of the protein.

On average, 1 L of LB medium yielded 20–25 mg of pure $HBx_{1\text{-}120}$ protein, with expression in M9 medium producing approximately 15–20 mg.

3.2. Purity and integrity of $HBx_{1\text{-}120}$

After the two-step dialysis, $HBx_{1\text{-}120}$ protein, while in 1.5M urea, can still be concentrated to at least 420 μM with a ratio of UV/Vis absorbances A_{260}/A_{280} of ~0.62, testifying the high purity of the protein sample (Fig. 5A). The same sample was analyzed by mass spectrometry (ESI-MS) after dilution to a protein concentration of ~1 μM and desalting. The protein monoisotopic mass is equal to 12,839.42 Da. After deconvolution (Fig. 5B), we found four molecular masses which are consistent with $HBx_{1\text{-}120}$ protein or modifications thereof: Peak 1 could be the intact protein with the monoisotopic mass of 12,837.38 Da. Peak 2 (12,707.36 Da) could correspond to the protein with its first methionine cleaved, which would have a monoisotopic mass of 12,708.38 Da [20,21]. Peaks 3 and 4, at 12,751.39 Da and 12,794.4 Da, respectively, could correspond to $HBx_{1\text{-}120}$ with a cleaved first methionine and a carbamoylation on top (addition of a $-CONH_2$ moiety). Carbamoylation is a natural reaction that can occur in high urea conditions. During the 48 h of IBs solubilization in 8 M urea there is an equilibrium between urea and ammonium cyanate. Cyanate ions can react with nucleophilic groups such as the ε-amino groups of lysine, which are particularly accessible to modifications in unfolded proteins. With one carbamoylation event and in the absence of the first methionine, a mono-isotopic mass of 12,751.39 Da and with two carbamoylation events a mass of 12,794.4 Da is expected, in very good agreement with the experimental masses for peaks 3 and 4. Hence, the MS analysis demonstrates that the obtained $HBx_{1\text{-}120}$ is not degraded and no unexpected modifications are detected which would affect activity or capacity to bind interaction partners.

3.3. Structural and functional analysis

To assess the conformational state of $HBx_{1\text{-}120}$, we recorded two amide correlation spectra on uniformly ^{15}N-labelled $HBx_{1\text{-}120}$ either in urea (1 M urea, Fig. 5C blue) or in a more native-like condition (residual 190 mM urea, Fig. 5C red). In both spectra, the overall signal dispersion in the ^{1}H dimension is low and centered around 8 ppm, indicating that $HBx_{1\text{-}120}$ is mostly in a random coil conformation in which secondary structure elements are not stably formed. The absence of evidence for pronounced secondary structure in the NMR spectrum recorded at near-native conditions suggests that $HBx_{1\text{-}120}$ is an intrinsically disordered protein or can exist as such [22,23].

Of note, in SDS-PAGE gels of pure and concentrated $HBx_{1\text{-}120}$ a faint band around 25 kDa is frequently observed (Fig. 3A). This band belongs to protein dimers, as corroborated by Western blot analysis (Fig. 3B). Indeed, dimerization of full-length HBx was reported earlier [19] and was ascribed to the presence of cysteine disulfide bonds, which seem to persist at a low level despite purification and dialysis conducted under reducing conditions and despite the presence of a reducing agent in the SDS-PAGE loading buffer.

Finally, in order to verify that recombinant $HBx_{1\text{-}120}$ after the removal of urea is functional, we established a pulldown assay with the known HBx interaction partner Spindlin1 under conditions resembling those of the NMR measurement (Fig. 6). In this assay, Spindlin1 is immobilized on a column with Ni-NTA resin through its histidine tag. $HBx_{1\text{-}120}$ can only be retained on the column after extensive washing if it

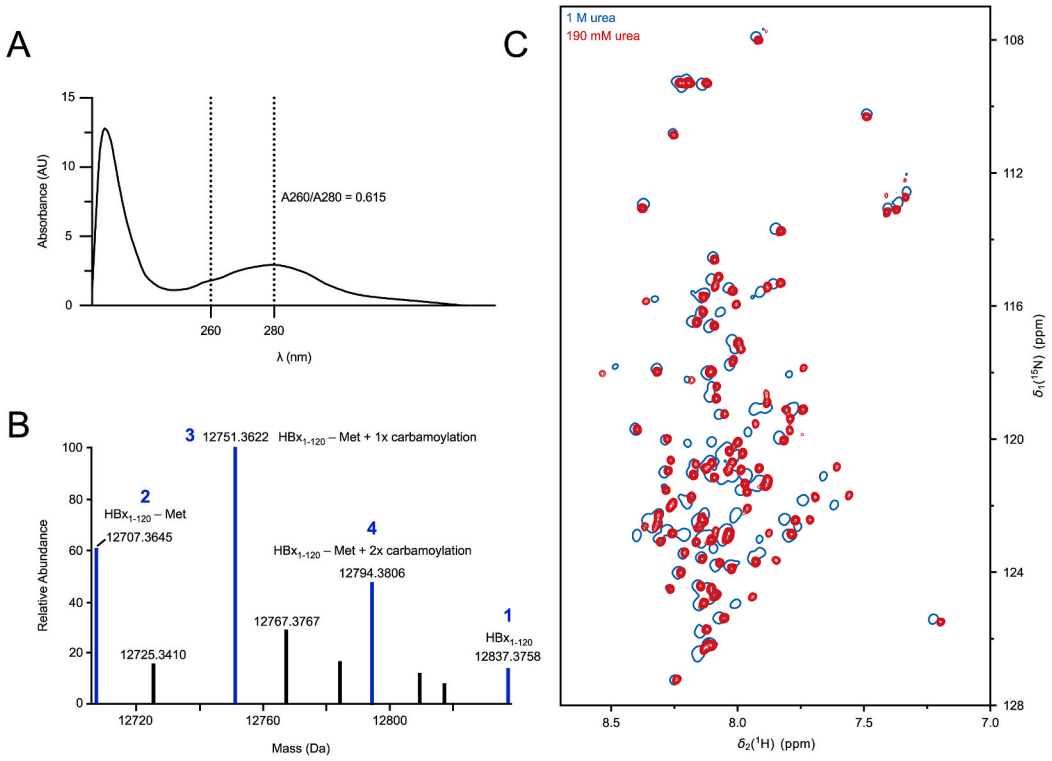

Fig. 5. A. UV/Vis curve obtained on a sample of $HBx_{1\text{-}120}$ concentrated to 420 μM (5.41 mg/ml) in 1.5 M urea, 1 M L-arginine, 150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM DTT. The ratio A_{260}/A_{280} is ~0.615, confirming that the sample is free from DNA contamination. **B.** Deconvoluted intact protein mass spectra showing the mass distribution in the $HBx_{1\text{-}120}$ sample after dilution. **C.** Overlay of two-dimensional NMR spectra (SOFAST-HMQC) showing the ^{1}H-^{15}N correlation of amide groups in the protein backbone with blue single contours showing the high molar urea condition (280 μM $HBx_{1\text{-}120}$ in 1 M Urea, 125 mM L-arginine, 150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM DTT) and in red contours showing the low molar urea condition (50 μM $HBx_{1\text{-}120}$ in 190 mM Urea, 125 mM L-arginine, 150 mM NaCl, 50 mM HEPES pH 7.4, 0.6 mM DTT). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

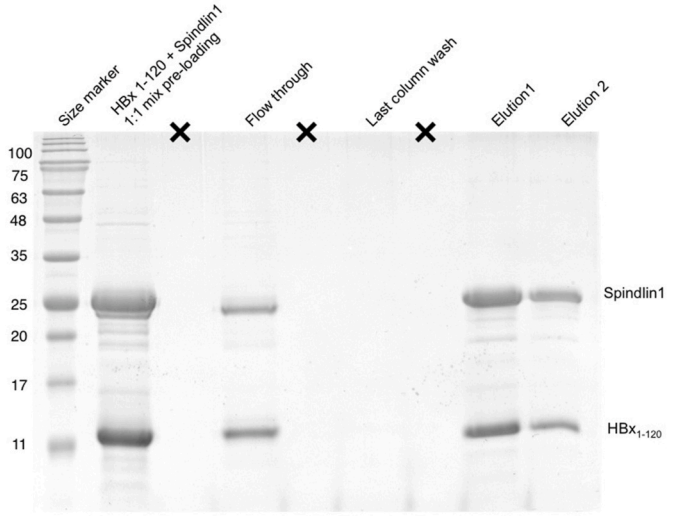

Fig. 6. Pulldown assay of his-tagged Spindlin1 protein immobilized on a Ni-NTA resin that binds to $HBx_{1\text{-}120}$. Lanes indicated with a cross are empty. In this assay his-tagged Spindlin1 (26.9 kDa) and $HBx_{1\text{-}120}$ (12.85 kDa) co-elute in an approximately equimolar fashion upon addition of elution buffer that detaches Spindlin1 from the resin, indicating the formation of a complex between recombinant Spindlin1 and $HBx_{1\text{-}120}$. Samples were loaded on an SDS-PAGE (18 %) gel.

is bound to Spindlin1. Upon addition of elution buffer supplemented with imidazole, his-tagged Spindlin1 and its binding partners are washed off together. We collected both Spindlin1 and $HBx_{1\text{-}120}$ in what appears to be an equimolar complex, according to the similar intensity of the elution bands in SDS-PAGE analysis (Fig. 6, lanes: Elution 1 + Elution 2). This experiment indicates that $HBx_{1\text{-}120}$, despite its disordered conformation and the presence of residual 190 mM urea and 125 mM L-arginine, can interact tightly with its partner protein Spindlin1.

4. Concluding remarks

In summary, we report a reliable method for expressing and purifying untagged $HBx_{1\text{-}120}$, a biologically relevant cancer-associated isoform of the HBV X protein. Our strategy overcomes solubility limitations by recovering the protein from IBs via urea solubilization and stepwise refolding in the presence of arginine. The resulting $HBx_{1\text{-}120}$ protein is of high purity and displays characteristics of an intrinsically disordered protein. The recombinant protein retains its ability to bind to Spindlin1, a known HBx interaction partner. Our method lays a foundation for structural and biochemical investigations of $HBx_{1\text{-}120}$ *in vitro*.

CRediT authorship contribution statement

Alexis Clavier: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Santiago Gómez-Evain:** Writing – review & editing, Investigation, Data curation. **Toshinobu Shida:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Rubaba R. Abanti:** Writing – review & editing, Methodology, Investigation. **Franziska Hammerstein:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Pavel Kielkowski:** Writing – review & editing, Methodology, Investigation. **Mila Leuthold:** Writing – review & editing, Methodology, Conceptualization. **Anne K. Schütz:** Writing – review & editing, Writing – original

draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Conflict of interests

The authors declare no competing interests.

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Data availability

Data will be made available on request.

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