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High-level expression and large-scale preparation of soluble HBx antigen from *Escherichia coli*

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The HBx (hepatitis B virus X protein) is a multifunctional regulator of cellular signal transduction and transcription pathways in host-infected cells. Evidence suggests that HBx has a critical role in the pathogenesis of hepatocellular carcinoma. However, the lack of efficient large-scale preparation methods for soluble HBx has hindered studies on the structure and function of HBx. Here, a new pMAL-c2x protein fusion and purification system was used for high-level expression of soluble HBx fusion protein. The high-purity fusion protein was obtained via amylose resin chromatography and Q-Sepharose chromatography. The untagged HBx was efficiently and rapidly purified by Sephadex G-75 chromatography after cleavage by Factor Xa at 23 °C. The purity of active HBx protein was >99 % with a very stable secondary structure dominated by α-helix, β-sheet and random structure. The purified HBx protein can be analysed to determine its crystal structure and function and its capabilities as an effective immunogen.

Introduction

Chronic infection with HBV (hepatitis B virus) is one of the major causes of HCC (hepatocellular carcinoma), and HBV-related HCC is among the top ten most common cancers worldwide. At present, approx. 400 million people suffer from chronic HBV infection, ensuring HBV's continued role as a major challenge to human health [1,2]. Although the pathogenesis of HBV-related HCC has not been fully described, evidence suggests that HBx (HBV X protein) plays a crucial role [3].

HBx is a multifunctional regulator that modulates transcription, signal transduction, cell-cycle progress, protein degradation pathways, apoptosis and genetic stability by directly or indirectly interacting with host factors [4]. Host proteins interacting with HBx are divided into three groups. The first group comprises intranuclear proteins, including general TFs (transcription factors), such as TFIIB, TFIIH, TFIIF, p120E4F and p53 and numerous specialized TFs [5–12]. The second group consists of DNA injury and repair-related proteins. HBx interferes with normal DNA repair by competitively binding to the DDB1 (damage-specific DNA binding protein 1) [13–15]. The third group is made up of intracytoplasmic proteins involved in the progress of signal transduction. HBx interacts with Pin1, the peptidylprolyl *cis–trans* isomerase that plays a critical role in normal signal-transduction-mediated cell functions, to accelerate hepatocarcinogenesis [16,17]. Moreover, HBx can impact the signal transduction pathway by binding with the SH3 (Src homology 3) motif of Rac1 to boost the replication of HBV in the hepatocytes [18]. HBx has also been shown to participate in host programmed cell death through its interactions with cFLIP {cellular FLICE [FADD (Fas-associated death domain)-like interleukin 1β-converting enzyme]-inhibitory protein} [19]. Taking these observations together, HBx is considered to be essential for maintaining HBV replication and has been implicated in the development of HCC in chronically infected patients.

Large-scale preparation of soluble HBx protein can provide a basis for detailed structural and functional studies and ultimately improve our understanding of HBx pathogenesis. However, previous attempts at large-scale production remain wholly ineffective. Use of *Escherichia coli* for growth and purification resulted in the whole HBx protein and/or a truncated version to be expressed in inclusion-body form [20–22]. Likewise, when HBx was expressed in a baculovirus system, cell fractionation experiments revealed that only a minor part of HBx was produced in a soluble form and most of the protein formed intracellular aggregates [23]. Analysis of ESI-MS (electrospray ionization MS) revealed that renatured HBx has only three disulfides, and it retains some functional properties, such as binding to RNA and p53 protein. This suggested that after denaturation and renaturation, the

Key words: amylose resin chromatography, *Escherichia coli* strain JM109, hepatitis B virus X protein (HBx), hepatocellular carcinoma, Q-Sepharose chromatography, Sephadex G-75 chromatography.

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; LTR, long terminal repeat; MBP, maltose-binding protein; TF, transcription factor.

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higher structure of HBx protein might be partially affected, whereas its primary structure remains intact. In the present study, a new pMAL-c2x fusion protein and purification system was developed for high-level expression of soluble HBx fusion protein without denaturation or renaturation. Moreover, in this system, HBx protein is completely expressed without any vector-derived amino acids at the N-terminus. This system will allow future research studies to determine the crystal structure of purified HBx protein.

Materials and methods

Reagents and supplies

Restriction enzymes and T4 DNA ligase were obtained from TaKaRa. Protein standards for molecular-mass determination, Xa protease and pMAL-c2x plasmid vector were purchased from New England Biolabs. Analytical-grade chemicals were purchased from Sigma.

Plasmid construction

The coding region of the HBx gene was derived from the plasmid pCR-XL-TOPO-HBV and cloned into the XmnI and HindIII restriction sites of the fusion-expression vector pMAL-c2x (NEB), to construct the pMAL-HBx recombinant plasmid. The sequences of primers P1 and P2 are 5′-ATGGCTGCTAGGCTGTGCTGC-3′ and 5′-CCCAAGCTTTTAGGCAGAGGTGAAAAAG-3′ respectively. HBx protein was verified for complete expression without any vector-derived amino acids at the N-terminus.

Expression and purification of fusion protein

The *E. coli* strain JM109 was transformed with the pMAL-HBx recombinant plasmid and grown overnight in LB (Luria–Bertani) broth plus 100 g/ml ampicillin at 37°C. The overnight culture was diluted 1:100 in fresh LB broth + ampicillin and grown at 37°C to a D_{600} (attenuance at 600 nm) of 0.6–0.7 and expression was induced with the addition of 0.03 mM IPTG (isopropyl β-D-thiogalactoside) for 2.5 h at 37°C. Bacteria were harvested by centrifugation at 4000 ***g*** for 10 min at 4°C and the bacterial pellets were suspended in column buffer [20 ml of 1.0 M Tris/HCl, pH 7.4, 11.7 g of NaCl, 2.0 ml of 0.5 M EDTA and 154 mg of DTT (dithiothreitol)]. The overnight culture stored at −20°C was thawed in cold water. Sonication was performed using an Ultra Cell™ (Sonics and Materials) at an output control of 25 W by continuous pulses (interstimulus interval: 10 s), interrupted by 5 s breaks on ice, with a 10 mm probe. The soluble and insoluble fractions of the *E. coli* lysate were separated by centrifugation at 12000 ***g*** for 15 min at 4°C. A total of 15 litres of cultured bacteria was used to collect the precipitate and purify ~60 mg/ml fusion protein using sonication, centrifugation, an affinity column and an ion-exchange column.

Factor Xa cleavage of fusion protein

The fusion protein was digested with Factor Xa *in vitro* at 1.0 mg/ml, using a substrate-to-enzyme ratio of 2% (w/w) for increasing periods of time (12, 24, 48 and 72 h) at 23°C. The cleavage reaction with Xa protease was carried out in deionized distilled water at pH 8.0.

Separation of fusion partner

The cleaved protein mixture was diluted with an equal volume of deionized distilled water and dialysed against 20 mM Tris/HCl, 25 mM NaCl, 0.5 M urea and 0.05% SDS (pH 9.5). The viral proteins were separated from the MBP (maltose-binding protein) partner on a Sephadex G-75 column (Amersham Biosciences), by using an FPLC system (Amersham Biosciences). The separated HBx protein was diluted with an equal volume of deionized distilled water.

SDS/PAGE and Western blotting

SDS/PAGE was used to analyse recombinant protein expression, the rate of protein purification and the proteolytic products of the MBP–HBx fusion protein after Xa protease treatment. Products were visualized via Coomassie Brilliant Blue staining. Western-blot experiments were carried out using polyclonal antibodies (Abcam) generated against recombinant HBx antigen and anti-rabbit IgG–HRP (horseradish peroxidase) as primary and secondary antibodies respectively. Visualization was performed with DAB · 4HCl (diaminobenzidine tetrahydrochloride) staining.

In vitro activity assay of HBx protein

MBP–HBx, HBx protein or control extract (protein concentration: 100 μg/ml) was added to 2 ml of DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FBS (fetal bovine serum), and then overlaid on a monolayer of LTR3CAT cells. After 6 h at 37°C, 8 ml of fresh DMEM with 10% FBS was added and the cells were harvested for the CAT (chloramphenicol acetyltransferase) enzyme assay [20] (Promega) after another 24 h.

CD analysis

CD spectra were scanned at the far-UV range (250–190 nm) with a CD spectropolarimeter (J-715; Jasco, Tokyo, Japan) in a 0.1-cm-pathlength quartz CD cuvette at 25°C. Protein concentration for the CD analysis was 50 μg/ml; 50 mM phosphate buffer (pH 6.5) was used to dissolve HBx protein. The values of scan rate, response, bandwidth and step resolution were 100 nm/min, 0.25 s, 1.0 nm and 0.2 nm respectively. Five scans were averaged to obtain one spectrum. The CD results were expressed in terms of mean molar ellipticity [θ] (degrees · cm^2 · dmol^{-1}).

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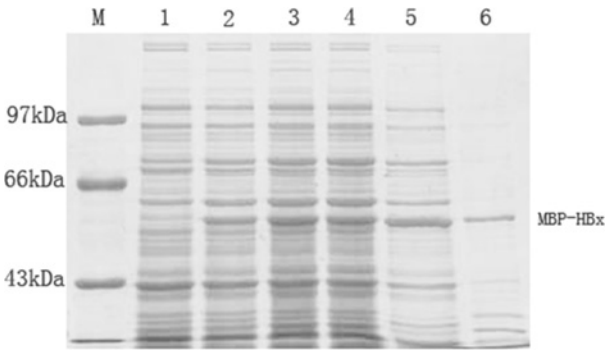

Figure 1 Electrophoretic analysis of the expression of pMBP-HBx recombinant plasmid in *E. coli*

Proteins were separated by SDS/10% PAGE and stained with Coomassie Brilliant Blue. Lane 1, uninduced JM109 cells harbouring the recombinant plasmid; lane 2, induced JM109 cells harbouring the recombinant plasmid after 1 h; lane 3, induced JM109 cells harbouring the recombinant plasmid after 2 h; lane 4, induced JM109 cells harbouring the recombinant plasmid after 3 h; lane 5, supernatant after sonication of the JM109 cells expressing MBP–HBx; lane 6, inclusion bodies. Lane M contains molecular-mass markers.

Results and discussion

Expression of MBP–HBx fusion protein

Up to 52% of the amino acids in the HBx protein are hydrophobic and the protein contains four disulfide bonds in a unique arrangement [22]. These structural characteristics may increase HBx protein's tendency towards intermolecular aggregation, improper folding and accumulation in the inclusion-body fraction. In order to study the crystal structure of HBx protein, we have used an efficient *E. coli* expression system to produce soluble HBx antigen in large quantities. pMAL-c2x vector is a powerful system for the expression of soluble proteins in *E. coli* because the pMAL-c2x vector has an MBP tag, which can increase the solubility of target proteins. The HBx gene was inserted into the XmnI site directly behind a Factor Xa cleavage site, which allowed HBx protein expression without any amino acid from the pMAL-c2x vector to remain after cleavage. The HBx protein was efficiently expressed in *E. coli* cells harbouring the pMBP-HBx recombinant plasmid as a fusion protein with MBP (Figure 1). As can be seen in Figure 1, lane 4, the MBP–HBx fusion protein with a molecular mass of approx. 59 kDa is detected as approx. 15% of the total bacterial cell proteins, and more than 90% of the protein can be found in the soluble fraction of the cell lysate (Figure 1, lane 5). Some attempts have been made to increase the quantity of the soluble form of the MBP–HBx protein (including lower incubation temperature for induction, mild disruption of the cells to avoid denaturation and aggregation of proteins, and use of detergent and thiol-group-containing reagent for solubilization), but none resulted in substantially increased solubility of the recombinant proteins.

Purification of MBP–HBx fusion protein by amylose resin chromatography and Q-Sepharose chromatography

An induced protein band of ~59 kDa was observed by SDS/PAGE (Figure 1, lanes 2–4) analysis of JM109 cells harbouring pMBP-HBx induced with 0.03 mM IPTG. After lysing the cells by sonication, the MBP–HBx protein primarily remained in the soluble fraction (~90%). The proteins were purified via amylose resin chromatography under specific conditions, yielding 40 ml of high-concentration (2 mg/ml) protein solutions (Figure 2A). Two protein bands representing fusion protein of ~59 kDa and MBP of ~42 kDa were observed by SDS/PAGE (Figure 2B). Correspondingly, two peaks were detected after purification via Q-Sepharose chromatography (Figure 3A) and each was confirmed by electrophoretic analysis (Figure 3B), suggesting that the HBx fusion protein was at least partially degraded in *E. coli* cells.

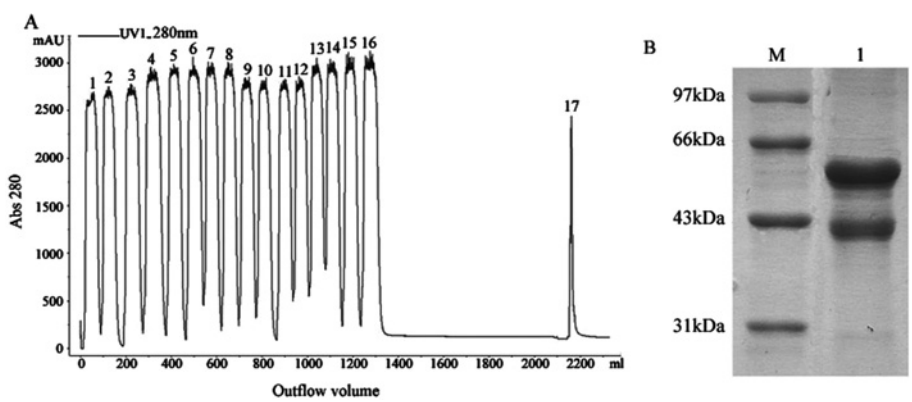

Figure 2 Purification of target protein by amylose resin chromatography

Proteins were separated by SDS/12% PAGE and stained with Coomassie Brilliant Blue. (**A**) Target protein was separated by amylose resin chromatography. The leftmost 16 peaks represent non-purified proteins in the bacterial lysate. Peak 17 on the far right represents the elution protein. Abs 280, A_{280}. (**B**) Electrophoretic analysis of the elution protein, including fusion protein of ~59 kDa and MBP of ~42 kDa.

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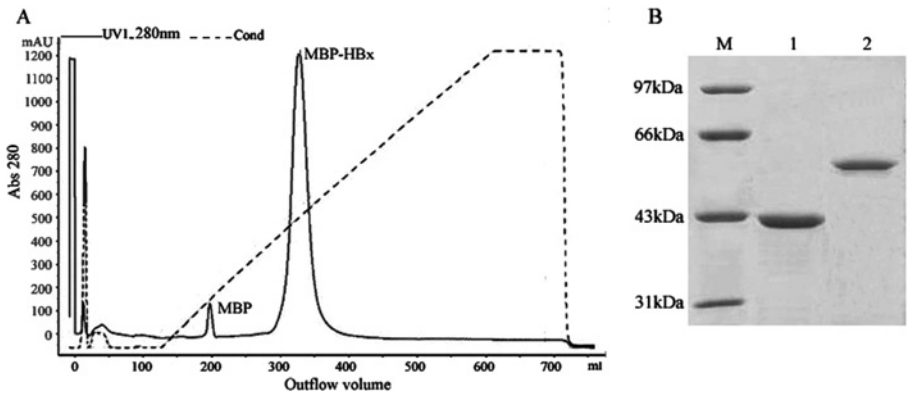

Figure 3 Purification of MBP and MBP–HBx by Q-Sepharose chromatography

Proteins were separated by SDS/12% PAGE and stained with Coomassie Brilliant Blue. (**A**) MBP and MBP–HBx were separated by Q-Sepharose chromatography. Abs 280, A_{280}; Cond, condition of iron exchange; mAU, milli-absorbance units. (**B**) Electrophoretic analysis of separated MBP and MBP–HBx (lane 1, MBP; lane 2, MBP–HBx).

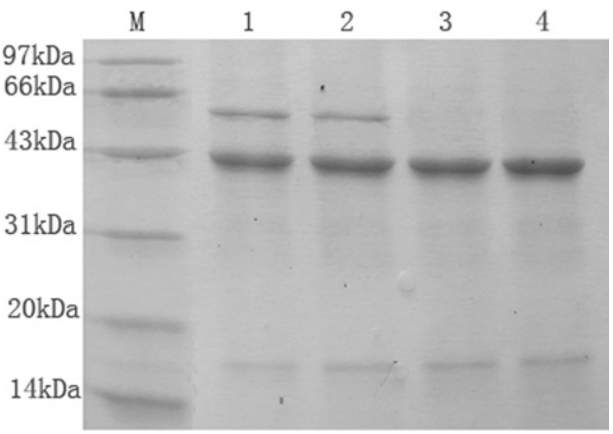

Figure 4 Cleavage of MBP–HBx fusion protein by Factor Xa

Proteins were separated by SDS/15% PAGE and stained with Coomassie Brilliant Blue. Lanes 1–4, cleavage products after 12, 24, 48 and 72 h.

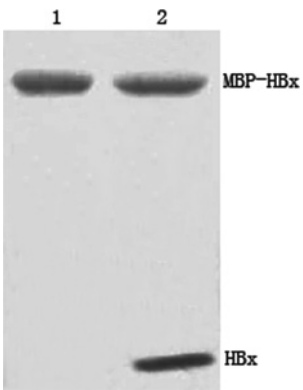

Figure 5 Western-blot analysis of purified MBP–HBx and HBx

Lane 1, purified MBP–HBx; lane 2, MBP–HBx cleaved by Factor Xa protease.

The concentration of the recombinant purified MBP–HBx protein in column buffer was determined and the purified protein was used in the UV cross-linking assay [24]. As a result of the selective solubilization, ~60 mg of soluble fusion protein was obtained from 15 litres of culture.

Cleavage of MBP–HBx fusion protein by Factor Xa

Cleavage with Factor Xa was carried out at a w/w ratio of 2% of fusion protein (e.g. 100 mg of fusion protein containing 2 mg of Factor Xa). Incubation of the fusion protein with Factor Xa demonstrated that the amount of the fusion protein decreases with time, concomitant with the appearance of cleavage products corresponding to MBP and HBx (Figure 4). Xa cleavage of the fusion protein was efficient and quantifiable after incubation for 48 h. The quality and quantity of the cleaved products did not change even after a 72 h incubation period. The advantageous conformation of the MBP–HBx fusion protein was reflected by the fact that the enzymatic reaction was mainly executed after a 48 h incubation period at 23 °C. The identity of the protein bands was confirmed by immunoblotting with anti-HBx rabbit serum (Figure 5) The cleaved HBx protein has no residual amino acids from the pMAL-c2x vector. This is advantageous for performing studies regarding the structure and function of HBx protein.

Separation of HBx protein from the fusion partner after cleavage by Factor Xa protease

After fusion protein cleavage by Factor Xa at 23 °C for 48 h, the solution became semi-opaque. Samples were subjected to centrifugation at 30 000 ***g*** for 20 min at 4 °C and the resulting supernatant and pellet were analysed by SDS/PAGE. Results showed that partial HBx was aggregated in the pellet. To overcome this problem, we dialysed the fusion protein cleavage mixture against 20 mM Tris/HCl,

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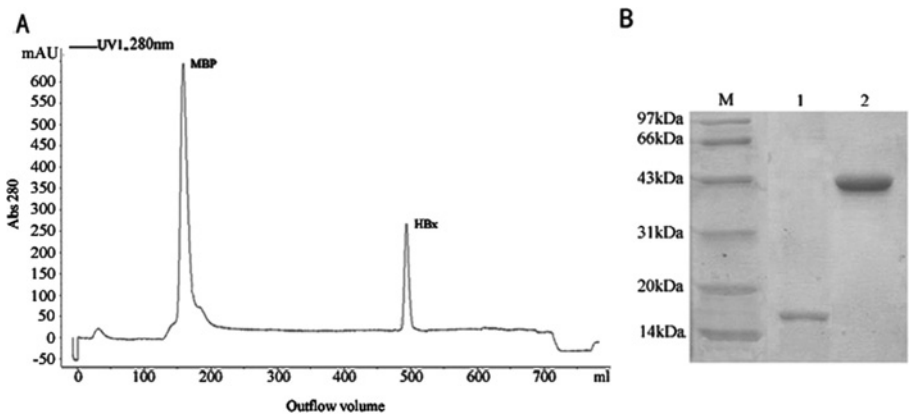

Figure 6 SDS/PAGE analysis of MBP and HBx separated by Sephadex G-75 chromatography

Proteins were separated by SDS/15% PAGE and stained with Coomassie Brilliant Blue. (**A**) MBP and HBx separated by Sephadex G-75 chromatography. (**B**) Electrophoretic analysis of separated MBP and HBx (lane 1, HBx; lane 2, MBP). Abs 280, A_{280}; mAU, milli-absorbance units.

25 mM NaCl, 0.5 M urea and 0.05% SDS (pH 9.5) at 4°C. This method is known to increase the solubility of HBx protein while avoiding denaturation processes [25]. After dialysis, the solution appeared clear. Since the experimental approach involving affinity purification of the cleaved MBP–HBx fusion protein was ineffective, we chose to separate the cleaved proteins according to their molecular masses. Cleaved MBP and HBx were separated on a Sephadex G-75 column (Figure 6A). The first chromatography run resulted in an efficient separation. Peak fractions were analysed for their purity by means of SDS/PAGE (Figure 6B). The two peaks shown in Figure 6(B) (lanes 1 and 2) represent those fractions primarily containing MBP and HBx at a purity of >99%. Using the above-outlined conditions, we obtained approx. 10 mg of recombinant HBx proteins.

In vitro activity assay of HBx protein

Previous studies have demonstrated that HBx protein from *E. coli* possesses biological activity [20]. A known function of HBx protein is *trans*-activation of particular *cis*-acting regulatory elements; of these, the HIV LTR (long terminal repeat) is the most comprehensively characterized. Thus we employed a HeLa cell-derived line (LTR3CAT) stably transfected with a plasmid containing the bacterial gene for CAT under the control of the HIV LTR sequences. This assay system has been described previously to directly measure cellular uptake of proteins [26]. Figure 7 shows the results obtained for CAT gene expression in the LTR3CAT cell line after overlay with DMEM, control extracts, MBP–HBx and HBx at a protein concentration of 100 μg/ml. Background CAT activity was detected in all lanes, albeit at a minimal amount (Figure 7, lane 1). Increases in CAT activity were clear in the MBP–HBx and HBx protein overlay lanes (Figure 7, lanes 3 and 4) compared with control extracts (Figure 7, lane 2). This level of stimulation by HBx is consistent with the results

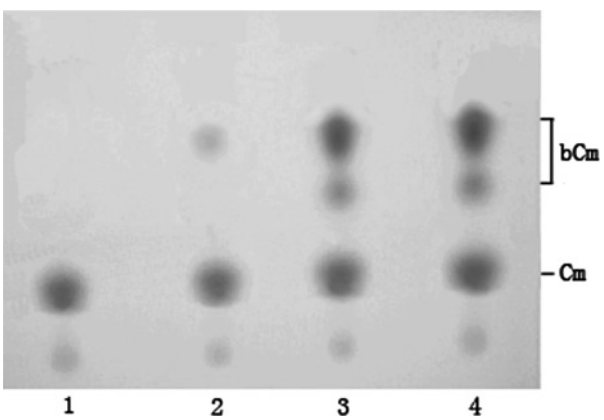

Figure 7 Functional activity of HBx protein

Lanes 1–4, cells overlaid with DMEM, control extracts, MBP–HBx and HBx at a protein concentration of 100 μg/ml respectively. Fold stimulation of CAT activity over the background is indicated (see the main text). Cm, chloramphenicol; bCm, butyrylated chloramphenicol.

obtained by others after DNA transfection [27]. Thus the HBx protein produced in *E. coli* was biologically active.

Previous findings indicated that HBx protein cannot bind directly with DNA sequences [28]. One possible mechanism to explain the *trans*-activating capabilities of the HBx protein in the absence of a DNA-binding function involves the HBx protein interacting with, or modifying, other TFs to indirectly activate transcription. For example, it has been confirmed recently that HBx can directly bind the VBP1 (von Hippel–Lindau-binding protein 1) TF to activate NF-κB (nuclear factor κB) gene transcription [29].

CD spectra of HBx protein

The far-UV CD spectra of a protein are a direct reflection of its secondary structure. Figure 8 shows the CD spectrum

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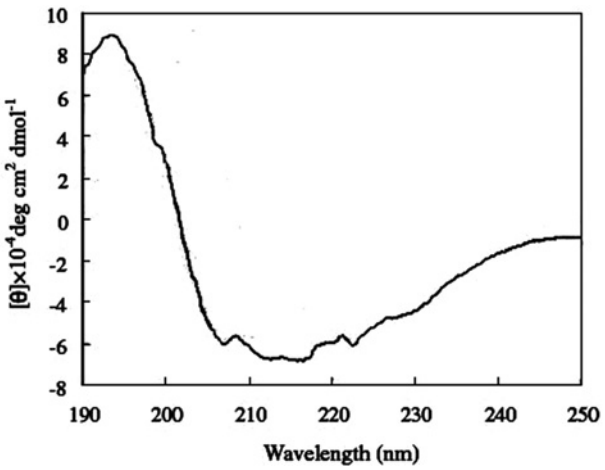

Figure 8 Far-UV CD spectrum of purified HBx protein

of purified HBx protein. The spectra of HBx protein revealed one strong positive peak in the vicinity of 194 nm, one negative shoulder at 218 nm, two negative troughs at 207 and 222 nm and one weak positive peak between 220 and 230 nm. The positive peak at 194 nm and negative shoulder at 218 nm are both indicative of the presence of β-sheet structure(s). The two negative troughs are caused by a negative Cotton effect characteristic of α-helical structure(s), and the weak positive peak between 220 and 230 nm is characteristic of a disordered protein structure [30]. The results obtained from the CD spectra analysis revealed that the secondary structure of HBx protein was composed of three different conformations including α-helix, β-sheet and random structure. This suggested that purified HBx protein in the absence of denaturation or renaturation processes remains a stable secondary structure in 50 mM phosphate buffer (pH 6.5) at 25 °C. Thus the purified HBx protein can be used for further analysis of its crystal structure and may form the correct crystal structure more easily under this condition.

We have described here a feasible *E. coli*-based method for high-throughput production and purification of recombinant MBP–HBx protein, a method that can yield an efficient separation of protein fusion partners. Furthermore, the resultant soluble HBx protein was obtained without denaturation or renaturation processes on a preparative scale. This procedure will benefit future studies that aim to determine the structure and function of HBx protein and will probably be helpful for the efforts to understand HBV pathogenesis.

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