PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 68 (1999) 119–127

The Journal of Steroid Biochemistry & Molecular Biology

Heterologous expression of wild type and deglycosylated human sex steroid-binding protein (SBP or SHBG) in the yeast, *Pichia pastoris*. Characterization of the recombinant proteins

L.M. Sui, J. Lennon [1], C. Ma, I. McCann, I. Woo, P.H. Pétra*

Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

Received 28 September 1998; accepted 7 November 1998

Abstract

Wild type, partially and fully-deglycosylated human sex steroid-binding protein (SBP or SHBG) cDNAs lacking the native eucaryotic signal sequence were cloned into a yeast expression vector containing the *Saccharomyces cerevisiae* α-factor for extracellular secretion. Following transformation into *Pichia pastoris*, the wild type and all constructed mutants were successfully expressed. The levels were lower for the deglycosylated mutants indicating that oligosaccharide side chains may play a role in SBP secretion. Under fermentation conditions, the wild type protein was expressed at a level of 4 mg/l while the fully-deglycosylated mutant T7A/N351Q/N367Q was expressed at about 1.5 mg/l. The latter was purified from several fermentation runs and was found to be completely deglycosylated, electrophoretically homogeneous and fully active. The aminoterminus was found to have the sequence NH_2QSAHDPPAV- indicating that cleavage of the α-factor occurred at the A^{+7}–Q^{+8} peptide bond. The molecular mass of the subunit was determined to be 39,717.8 Da, which is in complete agreement with the amino acid sequence of the T7A/N351Q/N367/Q mutant. The equilibrium constants for the dissociation of 5α-dihydrotestosterone and steroid binding specificity were found to be identical to that of the human plasma protein indicating that the missing N-terminal segment NH_2-LRPVLPT and the removal of oligosaccharide side chains do not affect the stability and active conformation of the protein. In conclusion, the data presented reveal that the SBP mutant T7A/N351Q/N367/Q is the protein of choice for solving the three-dimensional structure.

Keywords: Sex steroid-binding protein; SBP; Sex hormone binding globulin; SHBG; Deglycosylated SHBG; Testosterone; 17β-Estradiol; Yeast expression

1. Introduction

The sex steroid-binding protein, SBP (also referred to as sex hormone binding globulin, SHBG, and androgen binding protein, ABP), is a 93.4 kDa homodimeric glycoprotein which specifically binds testosterone (T) and 17β-estradiol (E_2) in plasma and testes [1–3]. Recent evidence indicates that the steroid binding site is located at the interface between the subunits and that dimerization is thus essential for the functioning of SBP as a steroid binding protein [2,4,5]. One gene codes for both SBP and ABP but its regulation is tissue-specific and the gene products differ primarily in the types of oligosaccharide side-chains and their sites of attachment. For many years, this laboratory has been interested in characterizing SBP for understanding the general and specific structural aspects of its steroid binding site and physiological role. The wild

Abbreviations: SBP, plasma sex steroid-binding protein; SHBG, sex hormone binding globulin; hSBP, human SBP; wphSBP, wild type *Pichia*-expressed hSBP; pdphSBP, partially-deglycosylated *Pichia*-expressed hSBP; fdphSBP, fully-deglycosylated *Pichia*-expressed hSBP; ABP, androgen binding protein; DHT, 5α-dihydrotestosterone; T, testosterone; E_2, 17β-estradiol; BMGY, buffered minimal glycerol-complex medium; BMMY, buffered methanol-complex medium; PAGE, polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +1-206-543-5714; fax: +1-206-543-6092.

[1] Present address: LSB Corp., 9620 Medical Centre Drive, Rockville, MD 20850-3338, USA.

0960-0760/99/$ - see front matter
PII: S0960-0760(99)00024-2

type amino acid sequence of human SBP was first determined by direct protein sequencing [6] and later deduced from partial and full-length human SBP/ABP cDNAs [7–10]. Expression systems for human SBP were developed in BHK-21 [10], COS-7 [11] and insect cells [12]. A full-length human SBP cDNA was also constructed by ligating a genomic restriction fragment [13] to a restriction fragment obtained from a naturally occurring mutant human SBP cDNA [14]. This cDNA contains a point mutation at position 801 [14] having cytosine instead of the wild type guanine (located at the corresponding position 728 of the cDNA sequence of Ref. [8], as well as in the codon for Leu-259 in the cDNA sequence of Ref. [7]). The guanine nucleotide is wild type because it is found in exon VII of the SBP gene sequence as determined independently in two laboratories [9,15]. The point mutation codes for a phenylalanine [14] in place of the wild type leucine at position 259 of the amino acid sequence [6]. Although expression of this mutant cDNA reveals interesting information [13,16,17], without additional data caution must be exercised when interpreting the properties of expressed protein from this construct, or others derived from it, as a paradigm for wild-type SBP. This concern stems from the fact that modification or substitution of a single amino acid in human SBP can have varying effects on its steroid binding activity ranging from none to complete loss of activity [11,18,19]. An amino acid substitution could likewise alter the protein conformation and weaken the interaction between the subunits or disrupt the receptor-binding site without having any discernable effect on the steroid-binding activity. In this context, it is interesting to note that a naturally occurring point mutation has recently been found in human SBP, which results in the amino acid substitution D327N [20]. This substitution results in the creation of an additional consensus site for *N*-glycosylation at position 327. That mutation represents an example of an amino acid substitution that has no effect on the steroid binding activity but has the important consequence of altering the metabolic clearance of SBP [21].

Progress on the elucidation of structure–function relationships in SBP has been slow due to inadequate supplies of pure protein. In order to undertake the solution of the three-dimensional structure of SBP, we therefore needed a new procedure to procure enough protein for setting up an undetermined number of crystallization trials. In addition, since microheterogeneity of the human protein arising from variability in carbohydrate content interferes in crystallization (unpublished data), development of an expression system that yields large amounts of fully-deglycosylated human SBP was imperative. Having been unsuccessful in over-expressing wild-type SBP-cDNA (unfused) in *E. coli* for the past year, we turned to yeast. In this paper, we report the construction of a human SBP cDNA mutated at carbohydrate attachment sites and its expression in *Pichia pastoris*, a methylotrophic yeast recognized to be an excellent host for the production of heterologous proteins [22–25]. This development led to a fermentation procedure that yields inexhaustible supplies of wild type, partially and fully deglycosylated SBPs. *Pichia pastoris* oxidizes methanol to formaldehyde using molecular oxygen and alcohol oxidase encoded in two genes, AOX1 and AOX2. The promoter of the former, which is tightly regulated by methanol, drives the major production of the enzyme and is therefore used for heterologous expression. The rationale for using *Pichia pastoris* are: (1) proteins are secreted into an almost protein-free medium, (2) post-translation modifications are similar to those in mammalian systems including proteolytic maturation, glycosylation and disulfide bond formation and (3) a repressing carbon source, such as glycerol, is not needed to prevent transcription from the AOX1 promoter. This latter characteristic makes the expression system easily adaptable to large-scale fermentation by allowing cells to metabolize glycerol to exhaustion before inducing with methanol. The *Pichia pastoris* strain GS115 contains a mutation in the histidinol dehydrogenase gene (*his*4) which prevents the cells from synthesizing histidine. Plasmid vectors have been engineered to contain the AOX1 promoter, the *HIS*4 gene complementing *his*4 for selection, the *Saccharomyces cerevisiae* α-factor signal sequence for secreting recombinant proteins into growth media and a kanamycin gene to allow screening for clones containing multiple gene copies. In this work, the signal sequence of human SBP cDNA was deleted and the construct was subcloned into such a vector. The data indicate that the *Pichia pastoris* expression system meets most of the criteria needed for pursuing aggressively elucidation of the remaining aspects of the structure and function of SBP.

2. Materials and methods

2.1. Materials

Pichia pastoris expression Kits, GS115 cells and pcDNA3 were purchased from Invitrogen (San Diego, CA). The QuickChange™ site-directed mutagenesis Kit, pBluescript II were purchased from Stratagene (La Jolla, CA). The Muta-Gene Phagemid Version 2 Kit was purchased from Bio-Rad (Richmond, CA). 5α-Dihydrotestosterone (DHT), testosterone (T) and 17β-estradiol (E_2) were purchased from Steraloids (Wilton, NH). Tritiated DHT, [1,2-^{3}H]DHT (58.4 Ci/mmol), was purchased from New England Nuclear (Boston, MA). Chemicals used in yeast expression

including fermentation were described in manuals provided by Invitrogen. ELISA Kits for SBP determinations were kindly provided by RADIM (Angleur, Belgium) and rabbit anti-goat IgG coupled to alkaline phosphatase used in Western blots was bought from Bio-Rad (Hercules, CA). The Amicon model S1 Spiral Cartridge used to concentrate yeast media was purchased from Millipore. All other equipment and chemicals used in this work were described in cited publications from this laboratory.

2.2. Buffers and solutions

BMGY: 1% yeast extract, 2% peptone, 4×10^{-5}% biotin, 1% glycerol. BMMY: BMGY in 0.5% methanol instead of 1% glycerol. All other stock solutions for growing *Pichia pastoris* are described in the Multi-Copy Expression Kit manual provided by Invitrogen.

2.3. DNA construction, screening and expression of recombinant SBP

A full-length hSBP cDNA was cloned in pBluescript II SK as previously described [10]. The human eucaryotic SBP DNA signal sequence ($Glu^{-28} \rightarrow Ala^{-1}$) was deleted from wild type pBlueScrp/sbp by site-directed mutagenesis according to Kunkel et al. [26] using the Version 2 Muta-Gene Phagemid kit and replaced with the yeast α-factor signal sequence according to the following procedure. Oligonucleotide primers were designed to keep the *Nco*1 site at the 5′ end necessitating the replacement of L^{+1} by V^{+1}, leucine was restored in the course of subsequent constructions. The resulting DNA (pBlueScrp/sbp$^-$) was linearized with *Nco*I to generate a template for PCR. The two primers were an antisense primer containing the *Asu*II site of sbp at nucleotide 226 and a sense primer containing the α-factor *Xho*1 site at the 5′ end. This second primer included an overhang sequence at the 5′ end coding for the α-factor cleavage signal SLEKR flanked to the SBP sequence *LRPVL* to yield SLEKR*LRPVL*. The resulting PCR fragment was digested with *Xho*1 and *Asu*II. Next, a restriction fragment was prepared by digesting SBP cDNA with *Asu*II and *Not*I. Finally, another restriction fragment was prepared by digesting pPIC9 with *Xho*I and *Not*I. The two restriction fragments and the PCR fragment were ligated to yield pPIC9/sbp$^-$. *Sac*I-linearized pPIC9/sbp$^-$ was electroporated into competent *Pichia* GS115 (*his*4$^-$) and selection was carried out at 30°C on MD plates (minimal dextrose medium, no his). Clones were picked and grown in 5 ml of BMGY to an OD of 3.0. The cells were harvested and resuspended in 5 ml of BMMY and 100% methanol was added to 0.5% every 24 h for 4 days to express SBP. The expression media were screened for SBP activity by steroid-binding analysis and ELISA. In an attempt to optimize signal cleavage and maximize SBP secretion, another construct was made to contain an EA repeat sequence, normally present at the C-terminal side of the cleaved peptide bond of the α-factor signal (SLEKR*EAEA*). This was accomplished by constructing and ligating the corresponding PCR fragment containing the *EAEA*$L^{+1}R^{+2}P^{+3}$ sequence with the other two restriction fragments, as described above. We did not expect that presence of the EAEA sequence at the N-terminus of SBP would affect the activity or folding of SBP. The three glycosylation sites of hSBP (Thr^7, Asn^{351} and Asn^{367}) were mutated together (T7A/N351Q/N367Q) or individually in various combinations using the QuickChange™ Site-Directed Mutagenesis Kit, as described in the manual by the manufacturer. DNA sequencing confirmed that only the desired mutations had occurred. To obtain multi-copy high expression recombinants, the wild type and the T7A/N351Q/N367Q mutant were subcloned into pPIC9K (containing kanamycin resistance) by inserting the *Sac*I–*Not*1 fragment of each pPIC9/sbp$^-$ into pPIC9K. *Pichia* GS115 cells were transformed by electroporation with pPIC9/sbp$^-$ and pPIC9K/sbp$^-$ linearized at *Sac*I. The transformants from pPIC9K/sbp$^-$ were screened with G418 (0–4.0 mg/ml). The yeast clones surviving 4.0 mg/ml G418 were chosen and further screened for SBP expression. For large-scale preparation, the high-level expression *Pichia* GS115 clones carrying the wild type and the T7A/N351Q/N367Q mutant sequences were expressed in 7-l cultures in a New Brunswick BIOFLO 3000 fermenter according to the instruction manual of Invitrogen.

2.4. Small-scale partial purification of recombinant SBPs

A small-scale purification procedure of (wild type *Pichia*-expressed hSBP), pdphSBP (partially-deglycosylated *Pichia*-expressed hSBPs) and fdphSBP (fully-deglycosylated *Pichia*-expressed hSBP) consisting of immunoadsorption on SBP-agarose [12,27] was used to determine their molecular weights by immunoblots. 50 ml of BMGY in 250-ml flasks were inoculated with a yeast colony and the cells were grown for 48 h, centrifuged and resuspended in 500 ml of BMMY in 2-l flasks. Expression was continued for 4 days and the media collected, concentrated to 3 ml and applied to anti-hSBP-agarose [27]. SBP was eluted with 0.2 M glycine, pH 2.2, and concentrated to 50–100 μl for Western blots.

2.5. Large-scale purification of wild type and fully deglycosylated SBP

wphSBP and fdphSBP expressed from the T7A/

Table 1
Expression levels in shaker flasks determined by ELISA and corroborated by charcoal binding assays. RBA = molar concentration of DHT causing a 50% reduction in [^{3}H]DHT binding/molar concentration of competitor causing a 50% reduction in [^{3}H]DHT binding × 100 [31]. O, indicates the position of the oligosaccharide side-chain O-linked to T7. N1, indicates the position of the oligosaccharide side-chain N-linked to N351. N2, indicates the position of the oligosaccharide side-chain N-linked to N367. wphSBP, wild type *Pichia*-expressed hSBP. fdphSBP, fully-deglycosylated *Pichia*-expressed hSBP. EAEA, is the amino acid sequence of the P1′–P2′–P3′–P4′ positions on the carboxy-terminal side of the scissile peptide bond (Schecter and Berger nomenclature [42]) preferred by the α-factor signal peptidase

Clones	Position of oligosaccharide side-chains	Expression (nM)	DHT K_d (nM)	RBA E_2(%)	RBA T(%)
wphSBP	---O-------N1---N2--	20	0.54	10.2	20.9
EAEA	EAEA ---O-------N1---N2--	6	0.60	9.3	14.4
N1N2	----------N1---N2--	8	1.07	6.7	12.1
ON2	---O ----------N2--	6	0.95	16.5	29.8
ON1	---O-------N1--------	7	0.96	2.6	7.5
N2	----------N2--	3	1.17	7.0	23.5
N1	----------N1--------	4	0.88	2.8	15.3
O	---O----------	4	0.58	11.2	33.5
FdphSBP	----------	5	0.70	12.0	28.2

N351Q/N367/Q mutant were purified to homogeneity from media collected from one to several 7-l fermentations by using a two-step procedure consisting of affinity chromatography on 5α-dihydrotestosterone-agarose followed by preparative gel electrophoresis. The growth media were first concentrated 10-fold with the Amicon Spiral Cartridge and subjected to affinity chromatography. The SBP was eluted with a DHT buffer and further concentrated to about 6 ml by ultrafiltration on Y10 Amicon membranes prior to preparative electrophoresis, as previously described for human plasma SBP [19,28,29]. Protein concentration was determined spectrophotometrically using $\varepsilon_{280} = 1.14 \times 10^5$ cm^{-1} M^{-1} [30].

2.6. Steroid binding analyses

Media from shaker flasks were screened using an SBP ELISA kit (RADIM, Belgium) and collected daily for 7 days to measure DHT binding activity by charcoal adsorption [11]. K_d's of DHT were determined by Scatchard analyses [12,29] at 4°C and pH 7.4, on media diluted to 1 nM SBP. Binding of E_2 and T were determined by a relative binding affinity assay [31].

2.7. Analytical electrophoresis and blotting methods

SBP electrophoresed in SDS slabs [32] were electroblotted onto nitrocellulose membranes with the Transblot apparatus (Bio-Rad) and probed with goat anti-SBP and rabbit anti-goat IgG coupled to alkaline phosphatase [10,12,32]. Polyclonal monospecific hSBP antibodies were affinity-purified from goat plasma by passing through a column of protein G-agarose followed by adsorption on hSBP-agarose [27].

2.8. Automated Edman degradation

Pichia-expressed hSBP was blotted on PVDF membranes and sequenced with an Applied Biosystems model 470 Sequencer with on-line phenylthiohydantoin analysis using published programs [33].

2.9. Mass spectrometry

The molecular weight of fdphSBP was determined by mass spectrometry as carried out by MALDI TOF-MS (matrix assisted laser desorption/ionization time-of-flight) on a PerSeptive Biosystems Voyager-Elite EL time-of-flight mass spectrometer [34,35]. The matrix was 2,5-dihydroxybenzoic acid. The molecular weight was calculated from seven different spectra, each produced from averaging 100 laser pulses.

3. Results

3.1. Expression of hSBP in Pichia pastoris

Table 1 compares expression levels of secreted wild type, partially and fully deglycosylated mutants in shaker flasks. The level of wphSBP clones expressed reached as high as 20 nM, whereas most of the pdphSBP and fdphSBP clones expressed at the level of 3–8 nM. In fermentation with optimal growth and induction conditions, the level is at least 2.5 times higher than in shaker flasks for both wphSBP (50 nM or 4.7 mg/l) and fdphSBP (12 nM or 1.1 mg/l). Interestingly, wphSBP clones containing the $EAEAL^{+1}R^{+2}P^{+3}$ sequence designed to optimize signal cleavage and maximize SBP secretion showed a decrease in secreted

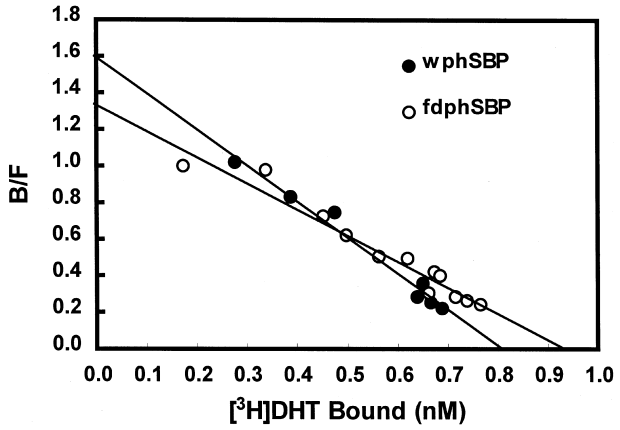

Fig. 1. Determination of the K_d for DHT binding to wild type (– ● — ● –) and fully deglycosylated hSBP (– ○ — ○ –) expressed in *Pichia pastoris* by Scatchard analysis. The calculated K_d's from these binding analyses are 0.54 nM for wphSBP and 0.70 nM for fdphSBP.

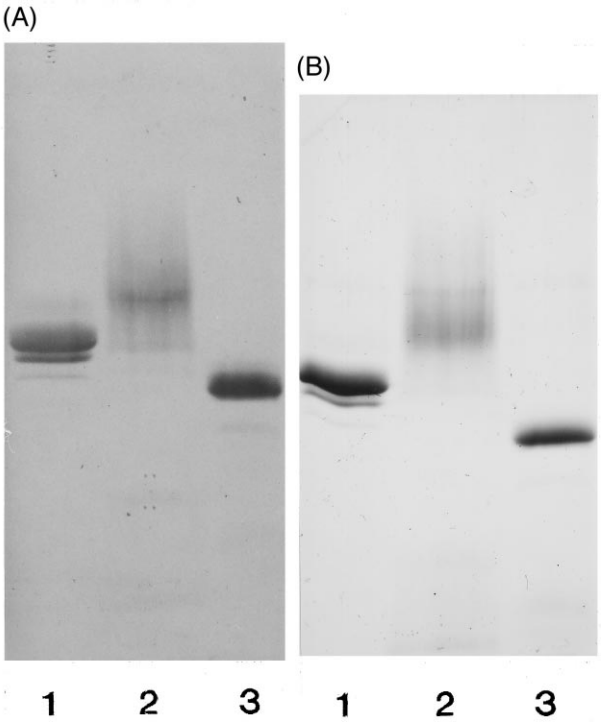

Fig. 2. Comparison of SDS-PAGE and immunoblot patterns of SBP purified from human serum and hSBPs expressed in *Pichia pastoris*. (A) Immunoblots; lane 1, plasma hSBP; lane 2, wphSBP; lane 3, fdphSBP. (B) SDS-PAGE stained with Coomassie blue; lane 1, plasma SBP; lane 2, wphSBP; lane 3, fdphSBP.

SBP in the medium, with a level similar to the deglycosylated clones. In order to search for clones containing multiple copies of the SBP gene, a total of 2000 of wphSBP transformants and 140 of fdphSBP generated from pPIC9K/sbp$^-$ were subjected to G418 screening. Only 22 of wphSBP and 4 of fdphSBP clones survived 4.0 mg/ml G418 and expressed higher SBP level. These clones were kept and used for large-scale expression. Most of G418 selected clones expressed a higher SBP level than that of non-selected, but some showed low or no expression. Expression time–course studies for all the clones showed that the level increased dramatically hours after initiation of methanol induction and reached their plateaus at day 4 (data not shown). The SBP activity in the collected medium remained constant for at least 4 weeks before purification.

3.2. Steroid-binding properties of Pichia expressed hSBP

The cDNAs coding for wild type, fully deglycosylated and partially deglycosylated SBPs were constructed and expressed in *Pichia pastoris* as described in Section 2. The DHT binding affinities were determined by Scatchard analysis and those of E_2 and T were estimated by competition analyses in samples taken from expressed media after 4 days of methanol induction. Fig. 1 shows a Scatchard plot for DHT binding to wphSBP and fdphSBP and Table 1 shows the relative binding affinity of E_2 and T. The results indicate that fdphSBP and all combinations of pdphSBP bind DHT equally as well as the wild type and exhibits the same steroid binding specificity in agreement with data obtained on fully deglycosylated hSBP generated enzymatically [32].

3.3. Purification and characterization of yeast expressed wild type and fully deglycosylated hSBPs

Purification of wphSBP and fdphSBP was achieved with a two-step procedure consisting of affinity chromatography on DHT-agarose followed by preparative PAGE [28]. The final yield of pure SBP obtained from a 7-l fermentation is about 10 mg for wphSBP and about 3 mg for fdphSBP. The immunoblot of Fig. 2(A) demonstrates that the proteins purified from fermentation experiments are SBPs because, like plasma hSBP, they both cross-react with anti-hSBP. The stained band in lane 3 migrates with a molecular mass of about 40,000 Da which is lower than the fully-glycosylated plasma hSBP monomer (lane 1, mass = 46,700 Da [6]). This mass difference is equal to the total mass of all three oligosaccharide side chains (6,200 Da [36]). The results thus indicate that the band in lane 3 in Fig. 2(A) is the fdphSBP monomer. Moreover, the data in Fig. 2(B), which shows a similar SDS gel but with 10 times the amount of protein applied for staining with Coomassie blue, demonstrate that fdphSBP is pure and that wphSBP exhibits a simi-

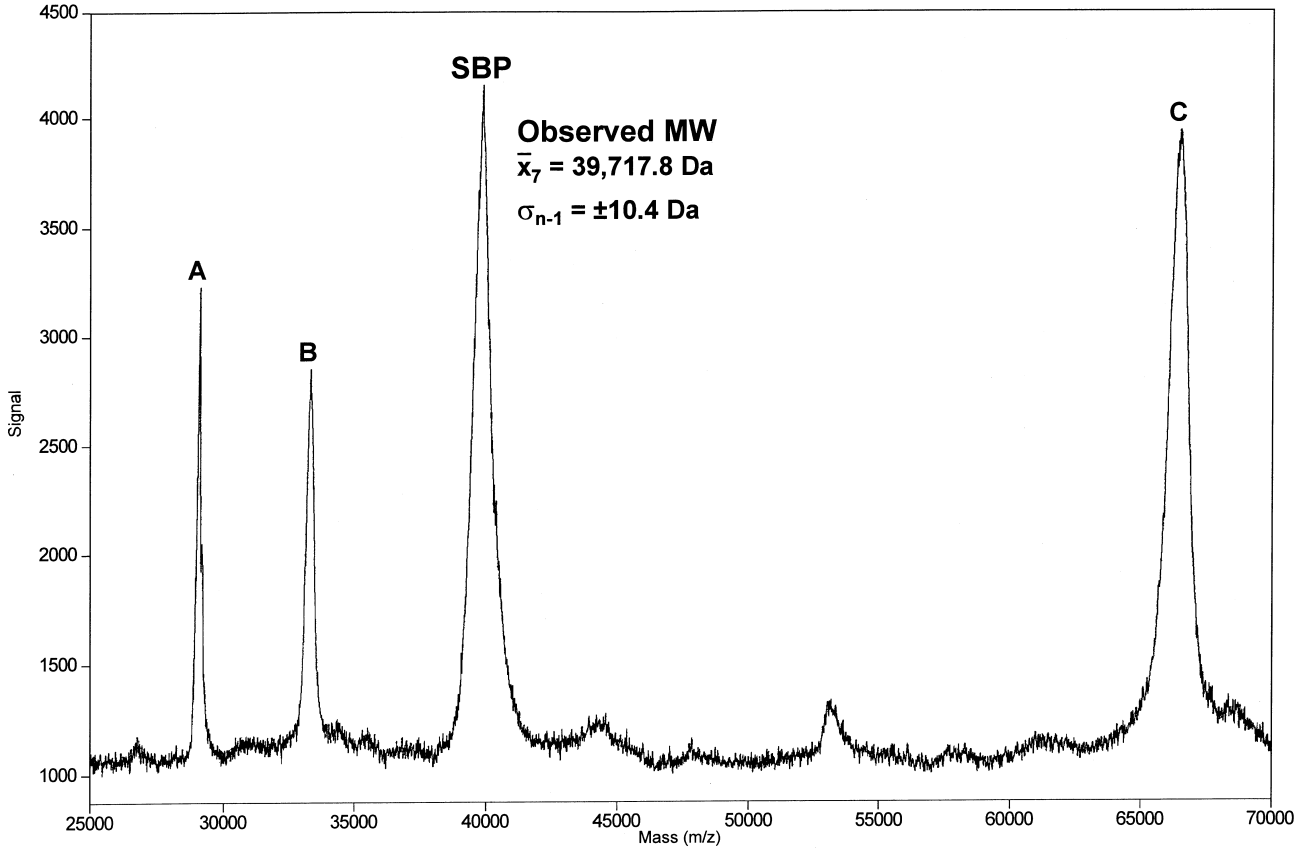

Fig. 3. Determination of the molecular weight of fully deglycosylated hSBP expressed in *Pichia pastoris* by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. The other peaks represent internal calibration standards: (A) Bovine carbonic anhydrase, $(M + H)^+ = 29{,}024.69$; (B) bovine serum albumin, $(M + H)^{2+} = 33{,}210.64$; (C) bovine serum albumin, $(M + H)^+ = 66{,}420.27$.

lar microheterogeneity as plasma SBP (lane 1). The higher molecular weight of wyhSBP suggests that *Pichia pastoris* hyperglycosylates SBP when compared to human liver.

An assignment of at least 15 amino acid residues was made at the aminoterminus of fdphSBP by amino acid sequence analysis. The results indicate the presence of a major component having the aminoterminus NH_2QSAHDPPAV– and a minor component (< 20%) having the aminoterminus NH_2RPVLPAQ-SAHDPPAV–. As shown in Fig. 3, the former yielded a molecular mass of 39,717.8 Da as determined by mass spectrometry. The other peaks in Fig. 4 are internal protein standards used for calibration. The minor component NH_2RPVLPAQSAHDPPAV– was not detected in the mass spectrometer indicating that its relative content in fdphSBP is much lower than estimated by automated Edman degradation. This finding is explained by the fact that much of glutamine at the aminoterminus of proteins is cyclized to pyroglutamic acid [37] which can no longer be alkylated by the Edman reagent. Thus, sequence analysis of fdphSBP by Edman degradation significantly underestimates the content of the major component NH_2QSAHDPPAV–; the relative amount of the minor component NH_2RPVLPAQSAHDPPAV– is then overestimated to about 5% of the total fraction of protein. The value of 39,717.8 Da, averaged over 7 measurements (each produced from averaging 100 laser pulses), essentially agrees with 39,719.3, calculated from the amino acid sequence of the fully deglycosylated polypeptide chain with aminoterminus NH_2-QSAHDPPAV– including the mutated amino acids. It is then clear that the T7A mutation in T7A/N351Q/N367Q gives rise to a new major cleavage site for *Pichia* signal peptidase at the A^{+7}–Q^{+8} peptide bond to yield SBP having an N-terminus of NH_2-QSAHDPPAV–. The data presented above conclusively prove that the T7A/N351Q/N367/Q mutant has Q at the N-terminus, is indeed fully deglycosylated and exhibits steroid-binding properties that are indistinguishable from those of the human plasma protein.

3.4. Correlation between α-factor cleavage and SBP secretion

Cells expressing wphSBP and fdphSBP were grown and induced with methanol as described above. Media

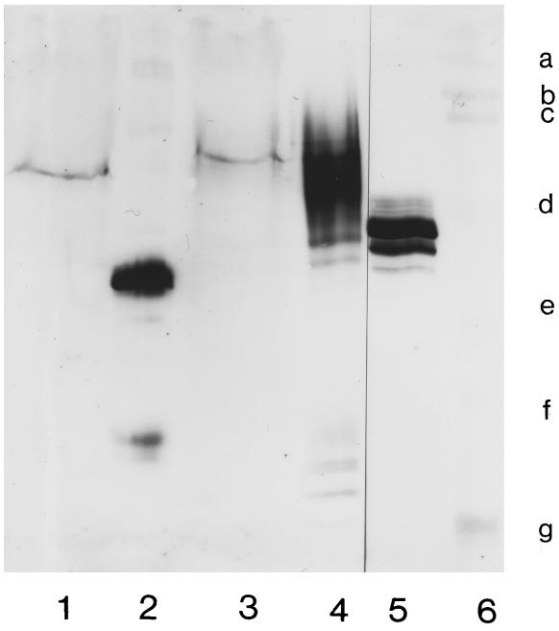

Fig. 4. Comparison of fdphSBP and wphSBP expressed intracellularly and extracellularly by Western blots. Lane 1, intracellular fdphSBP; lane 2, extracellular fdphSBP; lane 3, intracellular wphSBP; lane 4, extracellular wphSBP; lane 5, plasma hSBP; lane 6, molecular ladder of prestained SDS-PAGE standards (a) myosin, 203,000; (b) β-galactosidase, 115,00; (c) BSA, 83,000; (d) ovalbumin, 49,400; (e) carbonic anhydrase, 34,600; (f) soybean trypsin inhibitor, 29,000 and (g) lysozyme, 20,400. The bands in lane 6 of the standard molecular weight ladder (d), (e) and (f) were too faint to be seen after photography of the Western blot (none of the standards bands in lane 6 could be seen in the digital version of the figure). The lower bands in lanes 2 and 4 represent minor degradation products of SBP generated after one-year storage in 50% glycerol at 4 or −30°C.

were collected, cell pellets were homogenized with glass beads at 0°C and the molecular weights of expressed protein in cell extracts and in media were compared by Western blots. The results are shown in Fig. 4. Lane 1 shows the electrophoretic migration of the intracellular fdphSBP monomer and lane 2 that of extracellular fdphSBP. The molecular mass of intracellular fdphSBP, estimated from the ladder in lane 6, is about 55,000 Da whereas that of extracellular fdphSBP is 39,719 (see above). The difference as read directly from the gel, taking into consideration the log relationship between R_f and the molecular weight, is approximately 15,000 Da which corresponds well with the molecular mass of the α-factor (10,000 Da). Similarly, the approximate difference in molecular mass between intracellular wphSBP, 61,000 Da (lane 3) and the average of extracellular wphSBP, 50,000 Da (lane 4), also corresponds to the molecular mass of the α-factor. The Western blot also reveals that migration of the intracellular wphSBP monomer (lane 3) is slightly higher than that of intracellular fdphSBP (lane 1) reflecting a difference of about 6,000 Da, which is approximately equal to the mass of the oligosaccharide side-chains [36]. Finally, wild type secreted SBP (lane 4) has a slightly higher average molecular weight than plasma SBP (lane 5) indicating that SBP expressed in the *Pichia* system contains a higher sugar content. These data strongly suggest that the secretion of SBP in *Pichia* expression system is directly linked to the cleavage of the yeast α-factor.

4. Discussion

Heterologous expression of SBP has been attempted in a number of laboratories. The systems so far described yield protein levels that are too low and are not easily adaptable to large-scale preparation for pursuing experiments leading to SBP structure determination. Our best efforts at bacterial expression failed to yield recombinant SBP in the desired quantities (unpublished results); most of the expressed protein appeared as precipitate in inclusion bodies. Although glutathione *S*-transferase fused constructs of SBP have been expressed in bacteria [17], those exhibit steroid-binding affinities that are ten times lower than *Pichia*-expressed SBP, plasma SBP or unfused wild-type SBP expressed in other systems [1–3,10–12]. Fusing heterologous polypeptide chains to SBP is therefore likely to distort the native conformation making those constructs undesirable choices for pursuing structure–function studies. The best results were obtained with the baculovirus expression system, which yields SBP at levels of about 4.7 mg SBP/l in 6 days with K_d's for DHT and E_2 that are virtually identical to the human plasma protein [12]. That system, however, is tedious and expensive to scale up. Preliminary results for the expression of wphSBP in the *Pichia* system had produced similar levels as the baculovirus system (unpublished data). However, unlike insect cell expression, the yeast system is relatively inexpensive and adaptable to large-scale automated fermentation, as shown here. Although all constructs were expressed successfully, to date we have purified and characterized only wphSBP and fdphSBP, the latter being the ideal choice for crystallization trials. The results indicate that purification of the yeast recombinant proteins is much easier than those isolated from insect cell media. *Pichia* secretes very low levels of native proteins, thus the expressed protein represents the major protein fraction in the media allowing to reach purity with a simple two-step procedure. Immunoadsorption on polyclonal anti-SBP-agarose was also used for purifying yeast recombinant proteins, but disruption of the anti-SBP–SBP complex at low pH resulted in a 50% loss of steroid-binding activity, as in the case of SBP isolated from human serum (unpublished data). Therefore, that procedure probably should not be used for molecular characterization and physiological studies because the possibility

of undetectable conformational changes might affect the function of SBP. On the other hand, purification by affinity chromatography on 5α-dihydrotestosterone-agarose is much milder and ensures that the final product is active and uncontaminated with denatured or inactive SBP.

Characterization of fully deglycosylated SBP revealed an interesting finding. Creation of the T7A mutation in T7A/N351Q/N367Q generates a major cleavage site for *Pichia* signal peptidase at the A^{+7}–Q^{+8} peptide bond. This yields an SBP isoform having full steroid binding activity with aminoterminus NH_2-QSAHDPPAV– which had not been previously reported. Such a signal peptidase cleavage is consistent with those observed in more than 100 procaryotic and eucaryotic secretory proteins which are cleaved most often at small and neutral amino acid residues, alanine being the most common [38]. The cleavages previously reported at the N-terminus of SBP were L^{+1}–R^{+2} and P^{+4}–V^{+5} to yield NH_2-RPVLP– and NH_2-VLP–, respectively [6,39], and A^{-1}–L^{+1} to yield NH_2-LRPVLP– [40,41]. Since all these SBP isoforms exhibit full steroid-binding activity, we conclude that the native N-terminal segment NH_2-LRPVLPT is dispensable and is unlikely to play any role in the folding and dimerization of SBP. The segment is probably located on the surface of the protein and its removal is not expected to interfere with crystallization.

Wild type hSBP expressed in *Pichia* exhibits a higher molecular weight and a microheterogeneity similar to plasma hSBP. Although a detailed characterization of this microheterogeneity is not available at this time, the data indicate that, as in the case of human liver, yeast generates isoforms that vary in carbohydrate content. This is based on the fact that the fully deglycosylated SBP subunit T7A/N351Q/N367/Q is by contrast homogeneous and exhibits a lower molecular weight of 39,704, as determined by mass spectrometry. That value agrees with a molecular weight of 39,719, as calculated from the amino acid sequence of the mutated subunit polypeptide chain. Thus, the higher molecular weight and microheterogeneity of the wild type is likely to represent longer oligosaccharide side chains containing different amounts or types of monosaccharide units.

Partially and fully deglycosylated SBPs are fully active steroid binding proteins suggesting that the local structural changes resulting from the removal of oligosaccharide side chains have little effect, if any, on the active conformation of the protein, including the steroid binding site. This finding is particularly relevant to structural studies because the oligosaccharide side chains need to be removed for facilitating crystallization of the protein. The yield of wild type SBP in the *Pichia* expression system, designed to secrete protein into the medium, is four times that of the fully deglycosylated protein. These data suggest that the oligosaccharide side chains may play a role in the secretion of SBP in *Pichia* expression system and are likely to be important as well in liver secretion. This leads to the proposal that the oligosaccharide side chains may have two functions: (1) to decrease the metabolic clearance rate of the protein in human plasma, as recently shown [21] and (2) to assist in its secretion from liver.

In conclusion, the results presented in this paper establish *Pichia pastoris* as a eucaryotic cell host for expressing fully deglycosylated hSBP in sufficient quantities to complete studies leading to the elucidation of the structure and function of SBP. The results also yield information on the role of the oligosaccharide side chains by suggesting that they function in maintaining adequate concentrations of the protein in human plasma. Because estrogens, androgens and many drugs structurally related to these hormones are bound to SBP in plasma before they reach intracellular compartments of target cells, it is imperative to understand the structure of this protein for explaining the molecular basis of their binding specificities.

References

[1] U. Westphal, in: Steroid–Protein Interaction II. Monograph on Endocrinology, vol 27, Springer-Verlag, New York, 1986.
[2] P.H. Petra, The plasma sex steroid-binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology, and function, J. Steroid Biochem. Mol. Biol. 40 (1991) 735–753.
[3] D.R. Joseph, Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin, Vitamins Hormones 49 (1994) 197–280.
[4] L.M. Sui, W. Hughes, A. Hoppe, P.H. Petra, Direct evidence for the localization of the steroid-binding site of the plasma sex steroid-binding protein (SBP or SHBG) at the interface between the subunits, Protein Sci. 5 (1996) 2514–2522.
[5] K. Beck, T. Gruber, C.C. Ridgway, W. Hughes, L.M. Sui, P.H. Petra, Secondary structure and shape of plasma sex steroid-binding protein. Comparison with domain G of laminin results in a structural model of plasma sex steroid-binding protein, Eur. J. Biochem. 247 (1997) 339–347.
[6] K.A. Walsh, K. Titani, S. Kumar, R. Hayes, P.H. Petra, Amino acid sequence of the sex steroid-binding protein (SBP) of human blood plasma, Biochemistry 25 (1986) 7584–7590.
[7] B.G. Que, P.H. Petra, Characterization of a cDNA coding for sex steroid-binding protein of human plasma, FEBS Lett. 219 (1987) 405–409.
[8] S. Gershagen, P. Fernlund, A. Lundwall, A cDNA coding for human sex hormone binding globulin. Homology to vitamin K-dependent protein S, FEBS Lett. 220 (1987) 129–135.
[9] S. Gershagen, A. Lundwall, P. Fernlund, Characterization of the human sex hormone binding globulin (SHBG) gene and demonstration of two transcripts in both liver and testis, Nucl. Acid Res. 17 (1989) 9245–9258.
[10] F.S. Hagen, C. Arguelles, L.-M. Sui, W. Zhang, P.R. Seidel, S.C. Conroy, P.H. Petra, Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP). Characterization of the recombinant protein, FEBS Lett. 299 (1992) 23–37.

[11] L.M. Sui, A.W.C. Cheung, P.C. Namkung, P.H. Petra, Localization of the steroid-binding site of the human sex steroid-binding protein of plasma (SBP or SHBG) by site-directed mutagenesis, FEBS Lett. 310 (1992) 115–118.
[12] L.M. Sui, C. Wong, P.H. Petra, Over-expression of human sex steroid-binding protein (hSBP/hABP or hSHBG) in insect cells infected with a recombinant baculovirus. Characterization of the recombinant protein and comparison to the plasma protein, J. Steroid Biochem. Molec. Biol. 52 (1995) 173–179.
[13] W.P. Bocchinfuso, S. Warmels-Rodenhiser, G.L. Hammond, Expression and differential glycosylation of human sex hormone-binding globulin by mammalian cell lines, Mol. Endocrinol. 5 (1991) 1723–1729.
[14] G.L. Hammond, D.A. Underhill, C.L. Smith, I.S. Goping, M.J. Harley, N.A. Musto, C.Y. Cheng, C.W. Bardin, The cDNA-deduced primary structure of human sex hormone-binding globulin and location of its steroid-binding domain, FEBS Lett. 215 (1987) 100–104.
[15] G.L. Hammond, D.A. Underhill, H.M. Rykse, C.L. Smith, The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs, Mol. Endocrinol. 3 (1989) 1869–1876.
[16] W.P. Bocchinfuso, G.L. Hammond, Steroid-binding and dimerization domains of human sex hormone-binding globulin partially overlap: steroids and Ca^{2+} stabilize dimer formation, Biochemistry 33 (1994) 10622–10629.
[17] C. Hildebrand, W.P. Bocchinfuso, D. Dales, G.L. Hammond, Resolution of the steroid-binding and dimerization domains of human sex hormone-binding globulin by expression in *Escherichia coli*, Biochemistry 34 (1995) 3231–3238.
[18] C. Grenot, A. Montard, T. Blachere, M. Rolland de Ravel, E. Mappus, C.Y. Cuilleron, Characterization of Met-139 as the photolabeled amino acid residue in the steroid binding site of sex hormone binding globulin using Δ6 derivatives of either testosterone or estradiol as unsubstituted photoaffinity labeling reagents, Biochemistry 31 (1992) 7609–7621.
[19] P.C. Namkung, S. Kumar, K.A. Walsh, P.H. Petra, Identification of lysine-134 in the steroid binding site of the sex steroid binding protein of human plasma, J. Biol. Chem. 265 (1990) 18345–18350.
[20] S.G. Power, W.P. Bocchinfuso, M. Pallesen, S. Warmels-Rodenhiser, H. Van-Baelen, G.L. Hammond, Molecular analyses of a human sex hormone-binding globulin variant: evidence for an additional carbohydrate chain, J. Clin. Endocrinol. Metab. 75 (1992) 1066–1070.
[21] P. Cousin, H. Dechaud, C. Grenot, H. Lejeune, M. Pugeat, C. Baret, C. Brechant, Human variant sex hormone-binding globulin (SHBG) with an additional carbohydrate chain has a reduced clearance rate in rabbit, J. Clin. Endocrinol. Metab. 83 (1998) 235–240.
[22] K. Sreekrishna, R. Potenz, J. Cruze, W. McCombie, K. Parker, L. Nelles, P. Mazzaferro, K. Holden, R. Harrison, P. Wood, D.A. Phelps, C.E. Hubbard, M. Fuke, High level expression of heterologous proteins in methylotrophic yeast *Pichia pastoris*, J. Basic Microbiol. 28 (1988) 265–278.
[23] R.A. Brierley, R. Bussineau, R. Kosson, A. Melton, R.S. Siegel, Fermentation development of recombinant *Pichia pastoris* expression the heterologous gene: bovine lysozyme, Ann. N. Y. Acad. Sci. 589 (1990) 350–362.
[24] J.M. Cregg, T.S. Vedvick, W.C. Raschke, Recent advances in the expression of foreign genes in *Pichia pastoris*, Biotechnology 11 (1993) 905–910.
[25] C.E. White, M.J. Hunter, D.P. Meininger, L.R. White, E.A. Komives, Large-scale expression, purification, and characterization of small fragments of thrombomodulin: the roles of the sixth domain and of methionine 388, Protein Eng. 8 (1995) 1177–1197.
[26] T.A. Kunkel, Rapid and efficient site-specific mutagenesis without phenotypic selection, Proc. Natl. Acad. Sci. USA 82 (1985) 488–492.
[27] S. Bordin, P.H. Petra, Immunocytochemical localization of the plasma sex steroid-binding protein (SBP) in tissues of the adult male monkey, *Macaca nemestrina*, Proc. Natl. Acad. Sci. USA 77 (1980) 5678–5682.
[28] P.H. Petra, J. Lewis, Modification in the purification of the sex steroid-binding protein of human serum by affinity chromatography, Anal. Bioch. 105 (1980) 165–169.
[29] K.E. Mickelson, P.H. Petra, Purification and physico-chemical characterization of the sex steroid-binding protein of rabbit serum. Comparison with the human protein, J. Biol. Chem. 253 (1978) 5293–5298.
[30] P.H. Petra, P.C. Namkung, D.F. Senear, D.A. McCrae, K.W. Rousslang, D.C. Teller, J.B.A. Ross, Molecular characterization of the sex steroid-binding protein (SBP) of plasma. Re-examination of rabbit SBP and comparison with the human, macaque and baboon proteins, J. Steroid Biochem. 25 (1986) 191–200.
[31] B.J. Danzo, D.R. Joseph, Structure–function relationship of rat androgen-binding protein/human sex hormone binding globulin: the effect of mutagenesis on steroid-binding parameters, Endocrinology 135 (1994) 157–167.
[32] P.H. Petra, P.R. Griffin, J.R. Yates III, K. Moore, W. Zhang, The complete deglycosylation of native sex steroid-binding protein of human and rabbit plasma (SBP or SHBG). Effects on the steroid-binding activity, Protein Sci. 1 (1992) 902–909.
[33] M.W. Hunkapiller, R.M. Hewick, W.J. Dreyer, L.E. Hood, High-sensitivity sequencing with a gas-phase sequenator, Methods Enzymol. 91 (1983) 399–413.
[34] M.L. Vestal, P. Juhasz, S.A. Martin, Delayed extraction matrix-assisted laser desorption mass spectrometry, Rapid Commun. Mass Spectrum 9 (1995) 1044–1050.
[35] J.J. Lennon, K.A. Walsh, Direct sequence analysis of proteins by in-source fragmentation during delayed ion extraction, Protein Sci. 6 (1997) 2446–2453.
[36] G.V. Avvakumov, I.V. Matveentseva, L.V. Akhrem, O.A. Strel'chyonok, A.A. Akhrem, Study of the carbohydrate moiety of human serum sex hormone-binding globulin, Biochem. Biophys. Acta 760 (1983) 104–110.
[37] B. Blomback, Derivatives of glutamine in peptides, in: Methods in Enzymol, Vol.11, 1967, pp. 398–411.
[38] M.E. Watson, Compilation of published signal sequences, Nucl. Acids Res. 12 (1984) 5147–5156.
[39] P.H. Petra, F.Z. Stanczyk, D.F. Senear, P.C. Namkung, M.J. Novy, J.B.A. Ross, E. Turner, J.A. Brown, Current status of the molecular structure and function of the plasma sex steroid-binding protein (SBP), J. Steroid Biochem. 19 (1983) 699–706.
[40] P. Fernlund, C.B. Laurell, A simple two-step procedure for the simultaneous isolation of corticosteroid binding globulin and sex hormone binding globulin from human serum by chromatography on cortisol-sepharose and phenyl-sepharose, J. Steroid Biochem. 14 (1981) 545–552.
[41] G.L. Hammond, P.A. Robinson, H. Sugino, D.N. Ward, J. Finne, Physicochemical characteristics of human sex hormone binding globulin: evidence for two identical subunits, J. Steroid Biochem. 24 (1986) 815–824.
[42] I. Schechter, A. Berger, On the size of the active site in proteases. I. Papain, Biochem. Biophys. Res. Commun. 27 (1967) 157–162.