CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

The cleavage site specificity of human prostate specific antigen for insulin-like growth factor binding protein-3

Eriko Okabe[a,*], Jun-ichi Kajihara[a], Yoshiko Usami[b], Kazuyuki Hirano[b]

[a] *Development and Research Laboratories, JCR Pharmaceuticals, 2-2-10 Murotani, Nishi-ku, Kobe 651-2241, Japan*
[b] *Laboratory of Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan*

Received 21 December 1998; received in revised form 17 February 1999; accepted 19 February 1999

Abstract The cleavage site of human insulin-like growth factor binding protein-3 by urinary prostate specific antigen was examined. human insulin-like growth factor binding protein-3 was incubated with urinary prostate specific antigen at 37°C and its proteolyzed fragments were separated by a reversed phase HPLC followed by N-terminal amino acid sequence analysis, demonstrating that the cleavage mainly occurred at Tyr-159. The synthetic peptide including Tyr-159 was also cleaved at the same site, although its reaction rate was relatively low. These results indicate that human insulin-like growth factor binding protein-3 is specifically cleaved at Tyr-159 by prostate specific antigen. human insulin-like growth factor binding protein-3 was previously reported to be cleaved at five sites including Arg-97, Arg-132, Tyr-159, Phe-173 and Arg-179 by another group, however, prostate specific antigen preparation is possibly contaminated by trypsin-like protease. In contrast, our purified urinary prostate specific antigen had only a chymotrypsin-like activity, demonstrating that prostate specific antigen has the high substrate specificity for human insulin-like growth factor binding protein-3.

© 1999 Federation of European Biochemical Societies.

Key words: Prostate specific antigen; Insulin-like growth factor binding protein-3; Proteolysis

1. Introduction

Prostate specific antigen (PSA) is a glycoprotein with a molecular weight of 33 000–34 000 containing approximately 7% (w/w) carbohydrate [1,2], which was purified from human seminal plasma [3,4] or prostatic tissues [1]. Recently, urinary PSA (uPSA) was purified from human urine and its detailed physicochemical properties were investigated to be almost identical with those of PSA from seminal plasma (sPSA) [5]. While, PSA preparations have been made with differences in the enzyme specificity. In one case, it had both a kallikrein-like and a chymotrypsin-like activity [6] and in others, its activity was restricted to either of them [7–9]. However, several PSA preparations are found to be contaminated by trypsin-like protease such as hK2 [10] and recombinant PSA has been reported to have only a chymotrypsin-like activity intrinsically [11–13]. Recently, we purified uPSA from human urine, which contained both a kallikrein-like and a chymotrypsin-like activity [5]. In this report, uPSA was purified exhaustively to have only a chymotrypsin-like activity.

Furthermore, PSA was previously identified as one of the insulin-like growth factor binding protein-3 (IGFBP-3) specific proteases [14,15]. In fact, seminal plasma was found to contain fragments of IGFBP-3 corresponding to those generated by PSA cleavage of IGFBP-3 in vitro [14]. These findings suggest that PSA may be an important modulator of IGF action by regulating the affinity of IGFBP-3 for IGF.

PSA is also well known as a clinical marker of prostate cancer or benign prostatic hyperplasia (BPH) and its serum value is useful for diagnosis of these diseases and monitoring its clinical stage, tumor volume and histological grade [16]. There is a hypothesis that the concentration of free IGF increases by IGFBP-3 degradation by PSA in prostate tissue and finally, it may contribute to abnormal prostate cell growth [14,15,17–19]. In order to investigate the regulatory mechanism of the IGF-IGFBP-3 system by PSA, it is very important to clarify the cleavage specificity of IGFBP-3 by PSA. In a previous report, the cleavage sites of IGFBP-3 by sPSA were demonstrated to be Arg-97, Arg-132, Tyr-159, Phe-173 and Arg-179 [15]. The authors say that three sites (Arg-97, Arg-132, Arg-179) are consistent with a kallikrein-like activity and two sites (Tyr-159, Phe-173) are consistent with a chymotrypsin-like activity of PSA. However, trypsin-like proteases are possibly contaminated in the PSA preparation purified from seminal plasma. In this report, the cleavage specificity of IGFBP-3 was investigated by the use of uPSA containing only a chymotrypsin-like activity and further, its specificity was also confirmed in a peptide level.

2. Materials and methods

2.1. Purification of prostate specific antigen from human urine

Human fresh urine was adsorbed to chitosan and the pass through fraction was precipitated with ammonium sulfate (60% saturation). The precipitate was dialyzed against 50 mM sodium phosphate buffer (pH 5.5) and it was applied to a TSK-gel CM-Toyopearl 650C (TOSOH) column (10×15 cm) equilibrated with 50 mM sodium phosphate buffer (pH 5.5). The PSA was eluted with 0.5 M NaCl in the buffer, concentrated and the buffer exchanged to 50 mM Tris-HCl containing 1 M ammonium sulfate (pH 8.0). The concentrate was applied to a TSK-gel Phenyl-Toyopearl 650S (TOSOH) column (2.5×20 cm), equilibrated with 50 mM Tris-HCl containing 1 M ammonium sulfate (pH 8.0) and eluted with a linear gradient of ammonium sulfate (1 M→0 M). After concentrating the PSA fraction, it was applied to a Sephacryl S-200 HR (Amersham Pharmacia Biotech) column (3×80 cm), equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl. After the PSA fraction was collected and concentrated, it was applied to a Con A-Sepharose (Amersham Pharmacia Biotech) column (1×5 cm), equilibrated with 50 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and eluted with 0.2 M α-methyl-D-mannoside in the buffer. The eluate was concentrated and ammonium sulfate was added to adjust its concentration to 1 M. It was applied to an Alkyl-Superose HR5/5 (Amersham Pharmacia Biotech), equilibrated with 50 mM Tris-HCl (pH 8.0) containing 2 M ammonium sulfate (buffer A) and eluted with a linear gradient of 50 mM Tris-HCl (pH 8.0) (buffer B). Separation conditions were as follows: flow rate: 0.5 ml/min, detection: absorbance at 280 nm, HPLC system: Shimadzu LC-10A, gradient program: 0–10 min: 0% B, 10–20 min:

*Corresponding author. Fax: (81) (78991) 4465.
E-mail: kajihara@jcrpharm.co.jp

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)00275-6

0–40% B, 20–35 min: 40% B, 35–45 min: 40–50% B, 45–55 min: 50–100% B. The PSA fraction was applied to a Cosmosil 5C18-AR-300 (0.45×15 cm, Nacalai Tesque) using 0.1% trifluoroacetic acid (TFA)-acetonitrile (AN) system. Conditions were as follows: flow rate: 1 ml/min, detection: absorbance at 280 nm, HPLC system: Shimadzu LC-10A, gradient program: 0–15 min: 0–32% AN, 15–25 min: 32% AN, 25–35 min: 32–40% AN, 35–40 min: 40–80% AN.

The PSA content was determined using MARKIT-M PA (Dai-nippon Pharmaceutical), an ELISA kit for PSA measurement. The total protein was determined by the method of Lowry et. al [20].

2.2. Measurement of the enzyme activity

For measurement of the enzyme activity, MeO-Suc-Arg-Pro-Tyr-pNA (S-2586) (Chromogenix) and Pro-Phe-Arg-pNA (S-2302) (Chromogenix) were used as chromogenic substrates. The substrates and PSA were diluted with 0.1 M Tris-HCl (pH 8.0) containing 0.05% Tween-20 (buffer C), which prevents the adsorption of PSA and does not inhibit the enzymatic activity of PSA. After 10 μl of the synthetic substrate solution (1 mM) and 32 μl of buffer C were pre-incubated at 37°C for 5 min, 8 μl of PSA solution (85 μg/ml) was added. The reaction mixture was incubated at 37°C for 60 min, followed by adding 100 μl of 0.6 M AcOH to stop the reaction. Absorption of free *p*-nitroaniline at 382 nm was measured with a UV spectrometer DU640 (Beckman).

2.3. Electrophoresis and Western immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [21] with a 10–20% gradient gel, Maltigel 10/20 (Daiichi Pure Chemicals). For Western immunoblotting, the gel was electroblotted to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with rabbit anti-IGFBP-3 serum (Upstate Biotechnology) and ,after washing, with HRP-conjugated anti-rabbit IgG (Promega) followed by developing with Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products).

2.4. Degradation of recombinant human IGFBP-3 by uPSA

Recombinant human IGFBP-3 (20 μg, Upstate Biotechnology) expressed in Chinese hamster ovary (CHO) cells was dissolved in 200 μl of 0.1 M Tris-HCl containing 0.05% Tween-20 and added to 2 μg of uPSA followed by an incubation at 37°C for 24 h. A small portion of reaction mixture was analyzed by Western immunoblotting and others were applied to a Cosmosil 5C18-MS (0.45×15 cm, Nacalai Tesque) using a 0.1% TFA-AN system, followed by the separation of degradation products. The conditions were as follows: flow rate: 1 ml/min, detection: absorbance at 220 nm, system: Gilson HPLC system, gradient program: 0–60 min: 0–40% AN. Each peak was collected and its N-terminal amino acid sequence was determined by a Protein Sequencer 476A (PE Applied Biosystems).

2.5. Degradation of recombinant human IGFBP-3 by sPSA

sPSA was purchased from Calbiochem, but the sPSA also degraded S-2302 slightly, so it was purified by reversed phase (RP)-HPLC as described in the final step of uPSA purification before it was used for the degradation of IGFBP-3. The degradation was performed by the method as shown in Section 2.3.

2.6. Peptide synthesis

Peptides described below were synthesized by use of a Peptide Synthesizer 432A (PE Applied Biosystems) and purified by RP-HPLC. Peptides: SP1 DSQRYKVDYE, SP2 DSQRYKVD, SP3 QRYKVDYE and SP4 QRYKVD

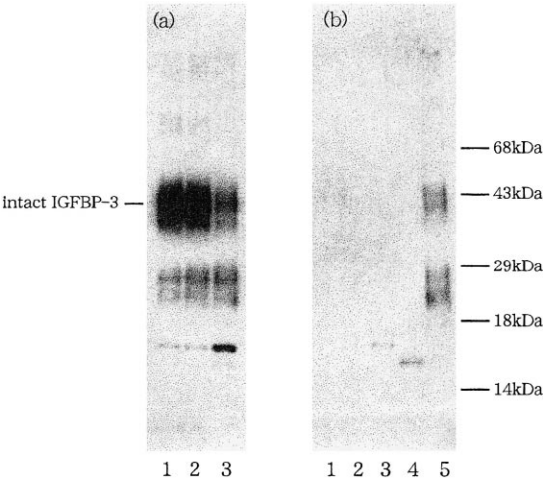

Fig. 1. The proteolysis of IGFBP-3 by uPSA analyzed by Western immunoblotting. (a) After the incubation of IGFBP-3 with uPSA at 37°C for 5 h (lane 1), 8 h (lane 2) and 24 h (lane 3). (b) After the separation by RP-HPLC. The lanes 1–5 correspond to each peak number 1, 2, 3, 4 and 6, respectively.

2.7. Degradation of synthetic peptide by uPSA

Synthetic peptide (50 nmol) and uPSA (8.7 μg, 0.26 nmol) were incubated at 37°C in 0.1 M Tris-HCl (pH 8.0) buffer containing 0.05% Tween-20. After 24 h, the reaction mixtures were applied to a Cosmosil 5C18-AR-II (0.45×15 cm, Nacalai Tesque) using the 0.1% TFA-AN system and the degraded peptides were obtained. Conditions were as follows: flow rate: 1 ml/min, detection: absorbance at 220 nm, HPLC system: Shimadzu LC-10A, gradient program: 0–24 min: 0–24% AN, 24–30 min: 24–100% AN, 30–35 min: 100% AN. The cleavage site was determined by amino acid composition analysis of degraded peptides.

3. Results and discussion

3.1. Purification of uPSA

Previously, we reported the purification and characterization of PSA from human urine [5]. In the report, we demonstrated that the physicochemical properties of purified uPSA were almost identical with those of sPSA and it had both trypsin-like and chymotrypsin-like activities. However, recently, some of the sPSAs were found to be contaminated by hK2, which is a member of the kallikrein family and has a trypsin-like activity [22], and PSA has been reported to have a chymotrypsin-like activity but not a trypsin-like activity [9–11]. These results suggest that our purified PSA is also contaminated by trypsin-like proteases such as hK2. Then, we re-purified uPSA by the improved method.

The purification was achieved by six steps of column chromatography (Table 1). At the step of Alkyl-Superose HR5/5, almost all of the inactive PSAs, which had some internal

Table 1
Purification of PSA from human urine

Purification step	PSA assayed by ELISA a (mg)	Total protein b (mg)	Specific activity (a)/(b)	Recovery (%)
Ammonium sulfate precipitation	13.0	33986	3.84×10^{-4}	100.0
CM-Toyopearl 650C	8.31	1404	5.92×10^{-3}	63.8
Phenyl-Toyopearl 650S	4.83	266	0.0182	37.1
Sephacryl S-200 HR	3.92	74.3	0.0528	30.1
ConA-Sepharose	2.74	23.3	0.118	21.0
Alkyl-Superose HR5/5	0.78	5.0	0.158	6.0
Cosmosil 5C18-AR-300	0.49	3.3	0.146	3.7

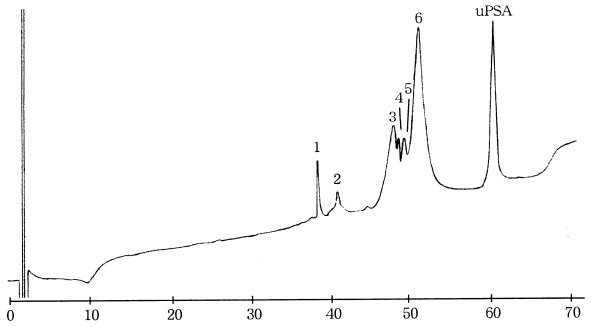

Fig. 2. The elution profile of proteolyzed IGFBP-3 by uPSA. After the incubation of IGFBP-3 with uPSA for 24 h at 37°C, the reaction mixture was separated by RP-HPLC.

cleavages [9] and migrated at a lower position than intact PSA on SDS-PAGE under the reduced condition, could be removed efficiently (data not shown). Some other protease activities were detected by enzyme assays using a chromogenic substrate after this step (data not shown). In order to remove other proteases, RP-HPLC was used as a final step. This procedure was successful for the purification of hK2 [22] because of its unusual resistance to the severe conditions in the presence of acetonitrile and 0.1% TFA. In the similar condition, uPSA was also stable and other contaminated proteases could be removed. As shown in Table 2, purified uPSA cleaved S-2586 but not S-2302, indicating that it has an only chymotrypsin-like activity.

As shown in Table 1, the PSA content in the final product measured by ELISA was low as compared with that by the Lowry method. The ELISA kits for PSA measurement are produced by many companies and the measured values are different [23]. In this case, the values measured by ELISA are estimated to be lower than the actual values.

3.2. Determination of the cleavage sites of IGFBP-3

IGFBP-3 was incubated with uPSA at 37°C and analyzed by Western immunoblotting after 5 h, 8 h and 24 h (Fig. 1a). Proteolyzed fragments increase in a time-dependent manner although its degradation rate was relatively low. The proteolyzed fragments were separated by RP-HPLC as shown in Fig. 2, followed by Western immunoblotting analysis (Fig. 1b). The amino acid sequence and the quantity of each fragment were determined as shown in Table 3. Peak number 1 and 2 contained the peptides which started from Arg-132 and Ala-98, respectively, but their amounts were small. Therefore, they could not be detected by Western immunoblotting (Fig. 1b). Peak number 3 and 4, the major degradation products, contained the peptides which started from Lys-160. It is conceivable that the difference in molecular weight of number 3 and 4 is due to the difference in sugar chain [24]. In peak number 6, none of the sequences except for N-terminal was detected by amino acid sequence analysis although Fig. 1b indicates that peak number 6 contained intact IGFBP-3 and

Table 2
Enzymatic activity of uPSA and sPSA

Substrate	uPSA	sPSA
Pro-Phe-Arg-pNA (S-2302)	N.D.	4.9
MeO-Suc-Arg-Pro-Tyr-pNA (S-2586)	20.5	17.9

Unit: pNA nmol/min/mg protein.

```
  0 GASSGGLGPV VRCEPCDARA LAQCAPPPAV CAELVREPGC GCCLTCALSE  50
 51 GQPCGIYTER GQPCGIYTER PDEARPLQAL LDGRGLCVNA SAVSRLRAYL 100
101 LPAPPAPGNA SESEEDRSAG SVESPSVSST HRVSDPKFHP LHSKIIIIKK 150
151 GHAKDSQRYK VDYESQSTDT QNFSSESKRE TEYGPCRREM EDTLNHLKFL 200
201 NVLSPRGVHI PNCDKKGFYK KKQCRPSKGR KRGFCWCVDK YGQPLPGYTT 250
251 YGQPLPGYTTS MQSK 264
```

Fig. 3. The cleavage sites of IGFBP-3 by uPSA (with an arrow).

a proteolyzed fragment. This result suggests that the fragment contained the residual portion after the release of the C-terminal fragment. From the above, it is suggested that uPSA cleaves IGFBP-3 carboxy-terminal of Tyr-159. Although a small amount was cleaved at Arg-97 and His-131, the rates of these cleavages are estimated to be 4.0% and 8.9% of total cleavages (39.2 pmol), respectively. Therefore, it is possibly considered that these cleavages at Arg-97 and His-131 are due to very small amounts of contaminated proteases which were not detected by the chromogenic substrate assay.

We also examined sPSA. The purchased sPSA was purified by RP-HPLC before use, because it had a slight trypsin-like activity (Table 2). The cleavage sites and proteolysis rate of sPSA were almost identical to those of uPSA (data not shown).

Previously, Fielder et. al. reported that the cleavage sites of IGFBP-3 (Fig. 3) by sPSA were Arg-97, Arg-132, Tyr-159, Phe-173 and Arg-179 [19]. They say that three sites (Arg-97, Arg-132, Arg-179) are consistent with a kallikrein-like activity and two sites (Tyr-159, Phe-173) are consistent with a chymotrypsin-like activity, but trypsin-like proteases are possibly contained in the sPSA preparation. The cleavage at Tyr-159 was identified by our experiment. It is considered that the cleavages at three Arg sites were due to contaminated trypsin-like proteases. The cleavage at Phe-173 was not detected in our experiment, however, this difference may mainly depend on the purity of PSA used in the experiment. It is possible that other proteases were contaminated in the sPSA preparation. As a minor possibility, the difference in IGFBP-3 is considered. We used glycosylated rIGFBP-3, expressed in CHO cells. On the other hand, they used a non-glycosylated one, expressed in *Escherichia coli*. The presence of sugar chains may influence the reactivity of PSA but a glycosylated IGFBP-3 is more preferable in consideration of the physiological condition.

3.3. Degradation of synthetic peptides

In order to confirm the cleavage specificity of uPSA, four peptides which contain the main cleavage site Tyr-159 were

Table 3
N-terminal amino acid sequence and the quantity of the peptide corresponding to each peak

Peak number	N-terminal amino acid sequence	Quantity (pmol)
1	R(132)VSPD	3.1
2	A(98)YLLP	7.0
3	K(160)VDYE	42.1
	A(98)YLLP	Trace
4	K(160)VDYE	9.1
5	G(1)ASSG	53.2
	K(160)VDYE	17.1
6	G(1)ASSG	163.3

The quantity was determined by N-terminal amino acid sequence analysis.

Table 4
The degradation rate of synthetic peptides by uPSA

Peptide	Sequence	Degradation rate (nmol/h)
SP1	DSQRYKVDYE	0.62
SP2	DSQRYKVD	0.17
SP3	QRYKVDYE	0.012
SP4	QRYKVD	Trace

Degradation rates are calculated from peak areas of peptides: D/(D+S)×50 (nmol)/24 (h). D, total area of degraded products; S, area of remained intact peptide.

synthesized (Table 4). Each peptide was incubated with uPSA at 37°C and after 24 h, the reaction mixtures were analyzed by RP-HPLC. Table 4 also shows the rates of peptides degraded by uPSA, which were calculated based on peak areas of degraded peptides. SP1 was the most specific for uPSA and SP3 and SP4 were hardly cleaved. In SP1, a Tyr residue corresponding to the position 163 was not cleaved. This result suggests that uPSA has a high specificity for the sequence around Tyr-159, although its degradation rate is relatively low. The results of SP2 ~ SP4 indicated that the two residues, Asp-Ser, are particularly important to the specificity of uPSA.

In this paper, the major cleavage site of IGFBP-3 by PSA was determined to be Tyr-159 and its substrate specificity was also confirmed by use of synthetic peptides. These results demonstrated that PSA had a high substrate specificity for IGFBP-3.

Recently, PSA has been considered to be one of the IGFBP-3 specific proteases and its involvement for the IGF-IGFBP-3 system has been supposed [14,15,17–19]. The PSA concentration in serum or prostate tissue increases in patients with prostate cancer or BPH. Therefore, there is a hypothesis that the concentration of free IGF increases by IGFBP-3 degradation by PSA in prostate tissue, resulting in abnormal prostate cell growth, which suggests that the block of proteolysis of IGFBP-3 by PSA may contribute to the improvement of prostatic disease such as prostate cancer or BPH. In this point, the amino acid sequence around Tyr-159, which we determined, may be utilized for the design of a specific inhibitor for PSA.

References

[1] Wang, M.C., Valenzuela, L.A., Murphy, G.P. and Chu, T.M. (1979) Invest. Urol. 17, 159–163.
[2] Wang, M.C., Loor, R.M., Li, S.L. and Chu, T.M. (1983) IRCS Med. Sci. 11, 327–328.
[3] Li, T.S. and Beling, C.G. (1973) Fertil. Steril. 24, 134.
[4] Sensabaugh, G.F. (1978) J. Forensic. Sci. 23, 106.
[5] Shibata, K., Kajihara, J., Kato, K. and Hirano, K. (1997) Biochim. Biophys. Acta 1336, 425–433.
[6] Watt, K.W.K., Lee, P.J., M'Timkulu, T., Chan, W.P. and Loor, R. (1986) Proc. Natl. Acad. Sci. USA 83, 3166–3170.
[7] Lilja, H. (1985) J. Clin. Invest. 76, 1899–1903.
[8] Akiyama, K., Nakamura, T., Iwanaga, S. and Hara, M. (1987) FEBS Lett. 225, 168–172.
[9] Christensson, A., Laurell, C.B. and Lilja, H. (1990) Eur. J. Biochem. 194, 755–763.
[10] Frenette, G., Gervais, Y., Tremblay, R.R. and Dube, J.Y. (1998) J. Urol. 159, 1375–1378.
[11] Kurkela, R., Herrala, A., Henttu, P., Nal, H. and Vihko, P. (1995) BioTechnology 13, 1230–1234.
[12] Takayama, T.K., Hujikawa, K. and Davie, E.W. (1997) J. Biol. Chem. 272, 21582–21588.
[13] Kumar, A., Mikolajczyk, S.D., Goel, A.S., Millar, L.S. and Saedi, M.S. (1997) Cancer Res. 57, 3111–3114.
[14] Cohen, P., Graves, H.C.B., Peehl, D.M., Kamarei, M., Giudice, L.C. and Rosenfeld, R.G. (1992) J. Clin. Endocrinol. Metab. 75, 1046–1053.
[15] Fielder, P.J., Rosenfeld, R.G., Graves, H.C.B., Grandbois, K., Maack, C.A., Sawamura, S., Ogawa, Y., Sommer, A. and Cohen, P. (1994) Growth Regul. 1, 164–172.
[16] Oesterling, J.E. (1991) J. Urol. 145, 907–923.
[17] Cohen, P., Peehl, D.M., Graves, H.C.B., Giudice, L.C. and Rosenfeld, R.G. (1994) J. Endocrinol. 142, 407–415.
[18] Peehl, D.M. (1995) Cancer 75, 2021–2026.
[19] Peehl, D.M., Cohen, P. and Rosenfeld, R.G. (1995) World J. Urol. 13, 306–311.
[20] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Frenette, G., Deperthes, D., Tremblay, R.R., Lazure, C. and Dube, J.Y. (1997) Biochim. Biophys. Acta 1334, 109–115.
[23] Kurihara, M., Akimoto, S., Akaza, H., Arai, Y., Usami, M., Imai, K., Tanaka, Y., Yamazaki, H., Kawada, Y., Koiso, K., Yoshida, O., Kotake, T., Yamanaka, H., Machida, T., Aso, Y. and Shimazaki, J. (1992) Jpn. J. Clin. Oncol. 22, 393–399.
[24] Firth, S.M. and Baxter, R.C. (1995) Prog. Growth Factor Res. 6, 223–229.