Proteolytic cleavage of recombinant two-chain factor VIII during cell culture production is mediated by protease(s) from lysed cells

The use of pulse labelling directly in production medium

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Abstract

During the production by mammalian cells of recombinant factor VIII from which the B domain was deleted (rFVIII), proteolytic cleavages in the C-terminal part of the heavy chain were observed (Kjalke et al., 1995). By radioactive pulse labelling it was investigated whether the cleavages took place inside the cells during protein synthesis or after release in the medium. The rFVIII-producing CHO (Chinese hamster ovary) cells were cultured in the presence of ^{35}S -methionine and then the cell lysate and the conditioned media were immunoprecipitated and analyzed by electrophoresis. By pulse labelling and chasing for various time periods, it was shown that the cleavages only took place after secretion of the protein from the cells. Adding cell lysate to uncleaved rFVIII caused cleavage of the heavy chain, as seen by loss of binding to a monoclonal antibody specific for intact rFVIII, indicating that the cleavage was performed by proteinase(s) released from the lysed cells. By incubating intact rFVIII with the multicatalytic proteinase (proteasome) present in cytoplasm and nucleus of eukaryotic cells, loss of binding to the monoclonal antibody was observed. This indicates that the multicatalytic proteinase, released from lysed rFVIII producing cells, could be responsible for the cleavage of rFVIII. Among several protease inhibitors tested, only bacitracin was found to diminish the extent of cleavage. Phosphatidylserine also protected rFVIII against cleavage, probably by binding to rFVIII.

Abbreviations: DMEM, Dulbecco's modified Eagles medium; NCS, newborn calf serum; rFVIII, recombinant coagulation factor VIII

Introduction

During development of a production process for recombinant proteins it is necessary to follow the product and impurity profiles and the quantity of the proteins. Assessment of quality usually requires purified protein. A method by which small volumes of production medium can be analyzed directly would thus be useful when optimizing process parameters. Pulse labelling of the production cell in the culture vessels followed by immunoprecipitation of the medium and analysis by SDS-PAGE and autoradiography is a very

versatile technique. Only microgram quantities of protein are needed when using radioactively labelled material. Analysis of the protein before secretion is made possible by lysing the cells.

Recombinant B-domain-deleted rFVIII is produced in a CHO cell line by coexpression of the genes for the heavy chain (the A1-A2 domains) and the light chain (the A3-C1-C2 domains) (Rasmussen et al., 1993). The heavy chain (92 kDa) and the light chain (80 kDa) are linked by a divalent cation, and the secreted two-chain molecule is fully coagulation active (Ezban et al., 1993). After thrombin activation the light chain is

found as a 73 kDa band and the heavy chain split into a 50 kDa band (corresponding to the A1 domain) and a 43 kDa band (corresponding to the A2 domain).

Previously, we identified two minor rFVIII forms which were cleaved in the C-terminal part of the heavy chain, i.e. the C-termini were at Glu720 or at Tyr729 instead of at the expected Arg740 (Kjalke et al., 1995). The present study uses pulse labelling techniques to elucidate when the cleavages of rFVIII took place. A possible candidate for the cleavage of rFVIII was identified, and it was investigated how the cleavage could be diminished thereby improving the overall yield of the process.

Materials and methods

Materials

rFVIII was purified as described previously (Kjalke et al., 1995). The monoclonal antibody F25 directed against the C-terminal part of the heavy chain (Kjalke et al., 1995) and polyclonal anti-factor VIII antibody were made in-house using standard procedures. Multicatalytic proteinase (EC 3.4.99.46) was purified from human placenta by Dorthe R. Pedersen and Klavs B. Hendil, University of Copenhagen. Protein A-Sepharose was from Pharmacia. Tosyl(4-toluenesulfonyl)-lysyl chloromethane, tosyl(4-toluenesulfonyl)-phenyl chloromethane, and benzyloxycarbonyl-phenylalanyl chloromethane were from Bachem, bacitracin (zinc free), soybean trypsin inhibitor and benzamidine were from Sigma, and D-phenylalanyl-prolyl-arginyl chloromethane was from Calbiochem. Human α -thrombin and all other proteinase inhibitors were from Boehringer Mannheim.

Cell culture

The CHO DG44 cell line (Urlaub et al., 1986), transfected with the genes for human factor VIII heavy chain (amino acid 1-740) and light chain (amino acid 1649-2332) on separate plasmids (Rasmussen et al., 1993), was grown at 37 °C (the cell growth phase). After lowering the temperature to 27 °C high amounts of active rFVIII was secreted into the production medium (the production phase). In the present experiments the cells were cultured in 9 cm² petri dishes in DMEM/Ham's F12 containing 10% NCS (Gibco) and 2.5 g l⁻¹ Soy protein hydrolysate (Sigma IV) during growth phase. During production phase 2% NCS, insulin (5 mg l⁻¹,

Novo Nordisk), Soy protein hydrolysate (2.5 g l⁻¹), ϵ -aminocaproic acid (0.25 g l⁻¹, Sigma), and heparin (2 U ml⁻¹, Novo Nordisk) were added to the basal medium. All pulse labellings were performed using 100 μ Ci ml⁻¹ of ³⁵S-methionine (Amersham) in production medium. Production medium was used in the chase periods.

Immunoprecipitation, electrophoresis and autoradiography

Cells harvested after pulse and chase were lysed in 20 mM TrisCl, pH 7.5 (Merck) containing 80 mM NaCl, EDTA (1 mM, Sigma), NP40 (0.5% w/v, BDH), sodium deoxycholate (0.5% w/v, Sigma), bovine serum albumin (1% w/v, Sigma), phenylmethanesulfonyl fluoride (1 mM, Sigma), pepstatin (1 μ g ml⁻¹, Sigma), and aprotinin (17 μ g ml⁻¹, Novo Nordisk) by freezing/thawing. The lysate was centrifuged and the supernatant used for further analyses. For experiments using rFVIII-containing medium, the medium was centrifuged to remove cell debris before addition of lysis buffer as described above. Samples were kept at -80 °C.

Any components in the samples binding non-specifically to Protein A-Sepharose were removed by precipitating with 5% (w/v) Protein A-Sepharose. Samples to be thrombin cleaved were incubated for 15 min with 2 U ml⁻¹ of human α -thrombin before immunoprecipitation with either a polyclonal anti-factor VIII antibody (0.25 mg ml⁻¹) or the monoclonal antibody F25 (0.1 mg ml⁻¹). The samples were incubated at 4 °C for one h with the antibody before addition of 10% (w/v) Protein A-Sepharose. The incubation was continued for one more hour, followed by isolation of the rFVIII/antibody/Protein A-Sepharose complexes by centrifugation. After washing, the pellets were boiled in SDS sample buffer (0.1 M TrisCl, pH 6.8 (Merck) containing 6% w/v SDS, glycerol (40% v/v), dithiotreitol (1.5% w/v), and bromophenol blue, all from Sigma. The samples were either analyzed immediately or frozen at -20 °C until use.

SDS-PAGE was performed using 10% (w/v) polyacrylamide gels (16 cm $\times$ 16 cm $\times$ 0.75 mm) run in a Hoefer vertical slab gel apparatus at 15 °C (Hames, 1990). After electrophoresis the gels were incubated for 30 min in 1 M sodium-salicylate (Sigma) for amplification of the signal, and dried in a Hoefer Drygel apparatus. The dried gels were placed on a RX Fuji X-ray film and left at -80 °C for exposure. Development was done by a Curix 60 AGFA machine. Each gel was

Table 1. Protease inhibitors tested for effect on cell-lysate mediated cleavage of rFVIII. For experimental details see **Materials and methods**

Inhibitor	Specificity of inhibitors	Concentration used	Effect
3,4-dichloroisocoumarin	serine proteases	2–50 μ M	–
Phenylmethanesulfonyl fluoride (PMSF)	serine and thiol proteases	0.05 mg ml ⁻¹	–
(4-aminidinophenyl)methane sulfonyl fluoride (APMSF)	serine proteases	0.05 mg ml ⁻¹	–
D-phenylalanyl-phenylalanyl-arginyl chloromethane (PPACK II)	serine proteases	0.01 mg ml ⁻¹	–
D-phenylalanyl-prolyl-arginyl chloromethane (PPACK)	serine proteases	0.01 mg ml ⁻¹	–
N-tosyl-lysyl chloromethane (TLCK)	serine proteases	0.1 mg ml ⁻¹	–
1,5-dansyl-glutamyl-glycyl-arginyl chloromethane (1,5-DNS-GGACK)	serine proteases	0.01 mg ml ⁻¹	–
N-tosyl-L-phenylalanyl chloromethane (TPCK)	serine proteases	0.1 mg ml ⁻¹	–
benzyloxycarbonyl-phenylalanyl chloromethane (ZPCK)	serine proteases	0.1 mg ml ⁻¹	–
benzamidine A	serine proteases	10 mM	–
benzamidine B	serine proteases	50 mM	–
soybean trypsin inhibitor	serine proteases	0.1 mg ml ⁻¹	–
aprotinin	serine proteases	0.01–1 mg ml ⁻¹	–
pefabloc SC	serine proteases	0.17 mM	–
antipain	papain, trypsin, cathepsin A and B	0.05 mg ml ⁻¹	–
elastitinal	serine proteases	0.1 mg ml ⁻¹	–
ϵ -aminocapronic acid	serine proteases	0.25 mg ml ⁻¹	–
leupeptin	serine and cystein proteases	0.5 μ g ml ⁻¹	–
E64	cystein proteases	0.02 mg ml ⁻¹	–
phosphoramidon	metallo proteases	0.4 mg ml ⁻¹	–
EDTA	metallo proteases	1.68 mg ml ⁻¹	–
phosphoamidon	metallo proteases	0.04 mg ml ⁻¹	–
bestatin	amino peptidases	0.04 mg ml ⁻¹	–
L-leucinethiol	aminopeptidases	0.05 mg ml ⁻¹	–
pepstatin	aspartate proteases	0.7 mg ml ⁻¹	–
calpain inhibitor I	cysteine proteases	0.1 mg ml ⁻¹	–
chymostatin	chymotrypsin	0.01–1 mg ml ⁻¹	–
eglin C	serine proteases	1–8 μ M	–
bacitracin	some serine and cysteine proteases, some aminopeptidases	0.01–0.1%	–
		1–5%	+

subjected to several exposures between one day and two weeks as needed.

In vitro cleavage of purified rFVIII

The cleavage of rFVIII was carried out *in vitro* by incubating 20 U ml⁻¹ (2 μ g ml⁻¹) rFVIII at 27 °C for the times indicated in Figure 3 with media containing 2×10^6 cells ml⁻¹, media containing lysate from the same number of cells (prepared by sonicating 5 min on ice), or in media without cells added. Sodium azide (0.02% w/v, Merck) was present during incubation. After centrifugation, the supernatant was loaded onto a F25-affinity column (Kjalke et al., 1995). The factor VIII activity in the flow-through fractions and the eluates was measured in a factor Xa-generation assay (Coatest FVIII, Chromogenix).

In order to inhibit the cell lysate-mediated cleavage of rFVIII, cell lysate from 2×10^6 cells ml⁻¹ was incubated with proteinase inhibitors (see Table 1) for one h at 22 °C with gentle shaking. rFVIII (20 U ml⁻¹, 2 μ g ml⁻¹) and 0.02% w/v sodium azide were then added and the incubation continued for additional three h at 27 °C. After centrifugation, the cleaved and uncleaved rFVIII in the supernatant were separated by F25-affinity chromatography. For some of the proteinase inhibitors (3,4-dichloroisocoumarin, bacitracin, aprotinin, E64, and pefabloc SC) the assay was also carried out with cell lysate from 0.5×10^6 cells ml⁻¹. All inhibitors were tested once, and inhibitors that gave more than 50% reduction in the amount of cleaved rFVIII were retested. Only if the inhibitor reduced the amount of cleaved rFVIII in three subsequent experiments, it was considered effective.

Table 2. Intact rFVIII was incubated with or without multicatalytic proteinase (MCP) as described in **Materials and methods** and the cleaved and uncleaved rFVIII were separated by F25-affinity chromatography (Kjalke et al., 1995). The factor VIII activity was determined in the starting material, the flow-through fraction containing the cleaved rFVIII, and the eluate containing the uncleaved rFVIII

Sample	Factor VIII activity (U)		
	Starting material	Uncleaved	Cleaved
rFVIII + MCP	47.4	21.1 (45%)	17.9 (38%)
rFVIII control	54.2	44.7 (83%)	3.9 (7%)

Figure 1. Autoradiography of rFVIII after electrophoresis. The pulse period was 4 h and the chase period 44 h. Lane 1 shows intact rFVIII and lane 2, thrombin-cleaved rFVIII. The bands correspond to the 92 kDa heavy chain (a), the 80 kDa light chain (b), the 73 kDa light chain after thrombin cleavage (c), and the 50 kDa A1 domain (d) and two variants of the 40 kDa A2 domain (e,f) released from the heavy chain by thrombin cleavage. The positions of standard proteins (in kDa) are shown on the right-hand side.

Cleavage of rFVIII by the multicatalytic proteinase was carried out by incubating the rFVIII (20 U ml^{-1} , $2 \mu\text{g ml}^{-1}$) with $9 \mu\text{g ml}^{-1}$ multicatalytic proteinase for 72 h at 27°C in 20 mM TrisCl, pH 7.5, containing 150 mM NaCl, 10 mM CaCl_2 , 10% v/v glycerol, and 0.02% w/v Tween 80, all from Merck (Table 2).

Results and discussion

The quality of rFVIII was followed during production by mammalian cells (CHO) of the rFVIII producing

cells by pulse labelling and immunoprecipitation of the growth media with a polyclonal anti-FVIII antibody. After electrophoresis and autoradiography, two bands arising from the A2 domains were seen (marked e and f on Fig. 1). The A2 domain with the highest mass came from rFVIII with intact heavy chain (amino acid 1-740), and the cleaved A2 domain with the lower mass came from rFVIII cleaved in the C-terminal part of the heavy chain during production (Kjalke et al., 1995). Two different rFVIII species with C-terminal truncation of the heavy chain were identified in our previous work, i.e. the C-termini were at Tyr729 and Glu720. However, only one of the truncated species was seen on Fig. 1, probably because the other rFVIII species was present in a concentration too low for detection without further purification.

We first examined the influence of the duration of the production phase at 27°C on the cleavage of the heavy chain. rFVIII producing cells were pulse labelled for 4 h followed by a 20 h or 44 h chase period, and rFVIII from cell lysates and media was immunoprecipitated with the monoclonal antibody F25 specific for rFVIII with an intact C-terminus of the heavy chain and analyzed by SDS-PAGE. Figure 2a shows the following autoradiogram when the chase period was 20 h. Intact A2 domain was seen in samples from medium (lanes 1 and 2) and from cell lysate (lanes 3 and 4). The thrombin-cleaved heavy-chain bands from lysates (lane 4) always had slightly higher mobility than those from medium (lane 2), probably due to immature glycosylation of rFVIII present inside the cells. After immunoprecipitation with F25, the remaining rFVIII in the supernatants was precipitated with a polyclonal anti-factor VIII antibody. In addition to residual intact A2 domain (e), cleaved A2 domain (f) was detected in the sample from the medium (lane 6). This band was not seen in samples from cell lysates, (lane 8) showing that the cleavage of the C-terminal part of the heavy chain took place after secretion from the cells.

When the chase period was extended to 44 h (Fig. 2b), only low amounts of labelled rFVIII in the medium was immunoprecipitated with F25 (lane 2) while large amounts of rFVIII containing a cleaved A2 domain was immunoprecipitated with the polyclonal antibody (lane 6). This indicates that most of the rFVIII molecules in the medium has been cleaved in the C-terminal part of the heavy chain during the 44 h chase period at 27°C . Only rFVIII with intact A2 domain was present inside the cells (lanes 4 and 8). Thus the longer chase period caused more heavy chain cleavage to take place in the medium. This cleavage

Figure 2. rFVIII from medium and cell lysate. The pulse period was 4 h and the chase period 20 h (a) or 44 h (b). Lanes 1, 2, 5 and 6 show rFVIII isolated from medium, and lanes 3, 4, 7 and 8 from cell lysate. Lanes 1–4 show samples immunoprecipitated with F25. Lanes 5–8 show the supernatants from samples in lanes 1–4 precipitated with the polyclonal anti-factor VIII antibody. Samples in lanes 2, 4, 6 and 8 were cleaved with thrombin prior to immunoprecipitation. Bands are as identified in the text to Figure 1.

could be mediated by cell surface bound proteases, by proteases present in the serum used in the production medium or by proteases released from cells upon death and lysis.

In order to determine the origin of the putative protease(s) responsible for the cleavage in the C-terminal part of rFVIII heavy chain, intact purified rFVIII was incubated with intact cells, lysed cells, or with fresh medium, followed by separation of intact and cleaved rFVIII by F25-affinity chromatography (Fig. 3). Half of the rFVIII incubated with cell lysate was cleaved within one hour and 70% was cleaved after 48 h. Incubating rFVIII with intact cells caused a slow increase in cleavage rate during the 48 h of incubation. No cleavage was observed in media without cells. This shows that the cleavage of rFVIII can be mediated by protease(s) released from dead and lysed cells. The slow cleavage rate by intact cells may be due to a slow rate of cell death and lysis at 27 °C causing intracellular proteases to be released. This was consistent with the observation, that the heavy chain cleavage was more pronounced after two days of chase at low temperature, i.e. the longer the cells were kept at sub-optimal conditions the more cells were lysed, and the more heavy chain cleavage took place.

The observed cleavage points in rFVIII heavy chain, i.e. at Glu720 and Tyr729 (Kjalke et al., 1995), were in accordance with some of the specificities reported for multicatalytic proteinase (Orlowski, 1990;

Rivett, 1993). The multicatalytic proteinase constitutes up to 1% w/v of the cell protein and is present in nucleus and cytoplasm of all eucaryotic cells where it takes part in intracellular protein turnover. Incubating rFVIII with purified multicatalytic proteinase caused an increase in cleavage of the heavy chain (Table 2). This shows that the multicatalytic proteinase released from lysed cells could be responsible for the cleavage of rFVIII during production.

Several inhibitors including well-known inhibitors of cellular proteinases (see Table 1) were tested for inhibition of the cleavage of rFVIII, however only bacitracin in concentrations higher than 1% w/v was found effective in the cell medium. Bacitracin A (the main component of commercial bacitracin) is a peptide consisting of twelve amino acids including D amino acids and ornithine with seven of the amino acids forming a ring structure (Toscano and Storm, 1982). Bacitracin is an antibiotic compound, but can also inhibit papain, subtilisin, and leucine aminopeptidase in a basically non-competitive manner (Mäkinen, 1972). The fact that trypsin is not inhibited by bacitracin, and that chymotrypsin is only inhibited slightly, made the author suggest that bacitracin only inhibits enzymes with a rather open active site that allows access of bulky molecules. Bacitracin also inhibits both the oxidative (Mizunaga et al., 1990) and reductive (Roth, 1981) activities of protein disulfide isomerase, and has been shown to inhibit the splitting of the two chains of insulin by

Figure 3. Cleavage of the rFVIII heavy chain by intact cells, cell lysate or medium. rFVIII was incubated with cells (●), cell lysate (▲) or media (■) as described in **Materials and methods** followed by separation of the cleaved and uncleaved rFVIII by F25-affinity chromatography. The ratio between the cleaved and the uncleaved rFVIII was calculated by measuring the factor VIII activity of the flow-through fraction (the cleaved rFVIII) and in the eluate (the uncleaved rFVIII) from the column.

this enzyme (Roth, 1981). This step has been hypothesized to be the first step in the degradation of insulin by liver cells (Varandani et al., 1972). FVIII contains several intradomain disulfide bonds and free cysteines, but no interchain disulfide bonds (McMullen et al., 1995). However, reduction of some of these disulfide bonds by protein disulfide isomerase might make the N-terminus of the heavy chain accessible for proteolytic enzymes. If this is the case, the effect of bacitracin could be due to inhibition of protein disulfide isomerase released from lysed cells. An other possibility is that bacitracin directly inhibits the proteolytic enzymes responsible for the cleavage. It is not known whether bacitracin is able to inhibit the multicatalytic proteinase.

The effect of protease inhibitors could also be measured directly in the production medium. After 4 h of pulse labelling, bacitracin was added at a final concentration of 1% w/v to the chase medium (Figure 4a). No

cleavage in the A2 domain was observed in the sample with bacitracin added.

Phospholipids are known to bind to factor VIII (Andersson and Brown, 1981; Gilbert and Drinkwater, 1993) and could thereby protect the protein against proteolytic degradation. When 0.025% w/v phosphatidylserine was added to the chase medium no cleavage of the A2 domain was observed (Fig. 4b). Both phosphatidylserine and bacitracin were slightly cell toxic at the concentrations used (data not shown) and were therefore only added during the chase period. The weak toxic effect could, of course, be expected to induce further proteolytic activity, but if so, the protective agents were still able to prevent cleavage of the FVIII heavy chain.

Whether the protective/inhibitory effect could be transferred to bioreactor scale is not known. Often the problems with proteolytic degradation in large scale bioreactors have been overcome by optimizing process

Figure 4. Phosphatidylserine and bacitracin prevented cleavage of rFVIII heavy chain. rFVIII-producing cells were pulse labelled for 4 h (37 °C) followed by a 44 h chase period (27 °C). In the chase period 1% bacitracin (a) or 0.025% phosphatidylserine (Sigma) (b) was added to the samples shown in lanes 1–2. Lanes 3 and 4 are control samples without inhibitors added. Samples shown in lanes 2 and 4 were thrombin cleaved prior to immunoprecipitation with polyclonal anti-factor VIII antibody.

parameters (Froud et al., 1991; Kratje et al., 1994) or choosing a cell line deficient in protease activity (Hosoi et al., 1991). However, the results illustrate that the pulse labelling techniques can be used to investigate problems of protein cleavage and help to find the possible solutions in recombinant production processes.

Concluding remarks

Only very small amounts of protein is needed for pulse chase experiments, i.e. a few micrograms of rFVIII in one ml of medium have been sufficient to observe the presence of different heavy chain species. By using a monoclonal antibody specific for the uncleaved rFVIII form and a polyclonal anti-factor VIII antibody it was possible to identify the intact and the cleaved species. The pulse labelling technique has enabled us to elucidate the time course of the cleavage of the heavy chain, and has proven useful for optimizing the quality of the produced rFVIII without time-consuming purification of the products.

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References

- Andersson L-O and Brown JE (1981) Interaction of factor VIII – von Willebrand factor with phospholipid vesicles. *Biochem. J.* 200: 161–167.
- Ezban M, Chapman B, Petersen J, Hansen K, Rasmussen PB, Thomsen J, Kjalke M and Kongerslev L (1993) Characterization of recombinant human two-chain factor VIII. *Thromb. Hemostasis* 69: 1088.
- Froud SJ, Clements GJ, Doyle ME, Harris ELV, Lloyd C, Murray P, Preneta PE, Stephens PE, Thomsen S and Yarranton GT: (1991) The development of a process for the production of HIV1 GP120 from recombinant cell lines. In: Spier RE et al. (eds) *Production of Biologicals from animal cells in culture*, pp. 110–114, Butterworth-Heinemann, Oxford.
- Gilbert GE and Drinkwater D (1993) Specific membrane binding of factor VIII is mediated by *O*-phospho-L-serine, a moiety of phosphatidylserine. *Biochemistry* 32: 9577–9585.
- Hames BD (1990) Preparation and electrophoresis of polyacrylamide gels. In: Hames BD and Rickwood D. (eds) *Gel electrophoresis of proteins. A practical approach*, pp. 30–50, Oxford University Press, Oxford.

- Hosoi S, Miyaji H, Satoh M, Kurimoto T, Mihara A, Fujiyoshi N, Itoh S and Sato S: (1991) Optimization of cell culture conditions for production of biologically active proteins. *Cytotechnology* 5: S17–34.
- Kjalke M, Heding A, Talbo G, Persson E, Thomsen J and Ezban M (1995) Amino acid residues 721–729 are required for full factor VIII activity. *Eur. J. Biochem.* 234: 773–779.
- Kobayashi N, Takenouchi T, Endo S and Munekata E (1992) ^1H -NMR study of the conformation of bacitracin A in aqueous solution. *FEBS Lett.* 305:105–109.
- Kratje RB, Lind W and Wagner R (1994) Evaluation of the proteolytic potential of in vitro-cultivated hybridoma and recombinant mammalian cells. *J. of Biotechnology*: 32: 107–125.
- Mäkinen KK (1972) Inhibition by bacitracin of some hydrolytic enzymes. *Int. J. Protein Res.* 4: 21–28.
- McMullen BA, Fujikawa K, Davie EW, Hedner U and Ezban M (1995) Location of disulfide bonds and free cysteines in the heavy and light chains of recombinant human factor VIII (antihemophilic factor A). *Protein Sci.* 4, 740–746.
- Mizunaga T, Katakura Y, Miura T and Maruyama Y (1990) Purification and characterization of yeast protein disulfide isomerase. *J. Biochem.* 108, 846–851.
- Orlowski M (1990) The multicatalytic proteinase complex, a major extralysosomal proteolytic system. *Biochemistry* 29: 10289–10297.
- Rasmussen PB, Nordfang O, Hansen K, Kongerslev L and Ezban M (1993) Recombinant two-chain factor VIII obtained in high amounts at decreased temperature. *Thromb. Haemost.* 69: 1086.
- Rivett AJ (1993) Proteasomes: multicatalytic proteinase complexes. *Biochem. J.* 291: 1–10.
- Roth RA (1981) Bacitracin: An inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase. *Biochem. Biophys. Res. Commun.* 98, 431–438.
- Toscano WA and Storm DR (1982) Bacitracin. *Pharmac. Ther.* 16: 199–210.
- Urlaub G, Mitchell PJ, Kas E, Chasin LA, Funanage VL, Myoda TT and Hamlin J. (1986) Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. *Somatic Cell and Molecular Genetics* 12: 555–566.
- Varadani PT, Shroyer LA and Nafz MA (1972) Sequential degradation of insulin by rat liver homogenates. *Proc. Natl. Acad. Sci. USA* 69, 1681–1684.

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