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Determination of recombinant hirudin structural deviants by capillary zone electrophoresis augmented with buffer additives

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Abstract

The polypeptide hirudin is a potent and specific thrombin inhibitor used in anticoagulant therapy and naturally occurring in medicinal leech. Using gene-technology methods, recombinant (r) hirudin can be produced on a large scale. Purity evaluation of the synthesized r-hirudin is essential to monitor co-expressed structural deviants and degradation products before therapeutic use. Although the well established RP-HPLC analysis appears to be the method of choice, in the case of r-hirudin baseline separation of the structural deviants is not necessarily achieved. Capillary zone electrophoresis augmented with buffer additives was used as a complementary technique to separate r-hirudin successfully from several similar species, in order to provide characterization information, as well as performing purity control and stability studies.

Keywords: Buffer composition; Stability studies; Precision; Hirudin; Proteins

1. Introduction

Hirudin from the leech *Hirudo medicinalis* is the most potent and specific known direct inhibitor of thrombin, the serine protease which functions in regulating hemostasis catalyzing the final steps in blood coagulation—the conversion of fibrinogen to clottable fibrin [1]. Large-scale production of the agent has proved difficult because of the limited availability of natural hirudin. Heparin, which forms a complex with antithrombin III (AT III), has been used to date for the prevention and treatment of thrombosis.

In recent years there has been a renewed interest in the application of hirudin as a therapeutic drug [2]. The advent of genetic engineering enables large quantities of recombinant hirudin (r-hirudin) to be produced in bacteria (*Escherichia coli*) or yeast cells (*Saccharomyces cerevisiae*), which secrete it into the surrounding culture medium. Concentration of the crude clarified supernatant is achieved using low-molecular-mass UF-membranes and conventional ion-exchange chromatography [3]. Purification of the final product is accomplished using ion-exchange and reversed-phase high-performance liquid chromatography (RP-HPLC) steps [4]. The sequence of the yeast hirudin has been determined by automated Edman degradation. It is 65 amino acids long, with a molecular mass of 6980 (Fig. 1). The hirudin amino acid composition includes a high content of asparagine and glutamine and their carboxylic acid forms; it lacks arginine, methionine and tryptophan. In contrast to the natural molecule which contains a sulfated tyrosine residue in position 63, lepirudin, a

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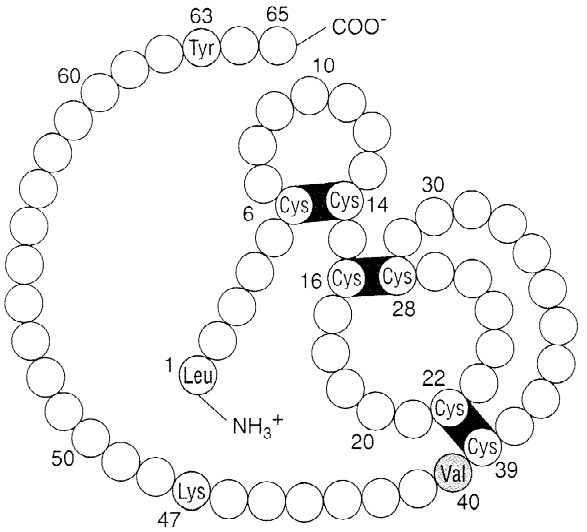

Fig. 1. Molecular model of recombinant hirudin lepirudin (Refludan).

desulfatohirudin secreted by transfected yeast cells, is not sulfated [5].

Furthermore it contains six cysteine residues and a COOH-terminal acidic portion which bears a stretch of homology with a thrombin cleavage site in prothrombin, and is consequently important for the binding to the recognition site of thrombin. Apart from lacking sulfation on Tyr-63, lepirudin differs from the natural hirudin in that it has leucine at position 1 (first N-terminal amino acid) rather than isoleucin, which results in an increase in the inhibition constant. Thus the hydrophobic nature of the N-terminal residues of hirudin is important for its interaction with thrombin [6].

Lepirudin (Refludan®, Aventis Behring, Marburg, Germany) is a potent anticoagulant and inhibitor of venous and arterial thrombosis. The clinical development of lepirudin as antithrombotic therapy has focused mainly on investigation of the drug in the treatment of unstable angina pectoris (UAP), for prophylaxis against and treatment of deep vein thrombosis (DVT), and in the treatment of heparin associated thrombocytopenia (HAT) type II [7–10].

Proteins, which have been cloned and expressed in large quantities in bacterial or yeast systems, need an extensive analysis of their purity before therapeutic application. Although purification by column chromatography generates high-purity products, low levels of degradation products, which are impurities from the biosynthesis do occur. Analysis of these variant forms has been enhanced by the utilization of capillary zone electrophoresis (CZE), a highly efficient technique which is increasingly used in the field of biotechnology [11–20]. Here we show that CZE, using a complex buffer system, enables the reproducible baseline separation of r-hirudin structural deviants.

2. Experimental

2.1. Samples

Purified [Leu^1,Thr^2]-63-desulfohirudin (lepirudin) produced in *S. cerevisiae* (Aventis Behring) was used in the development of the capillary zone electrophoresis method. The method was also applied to r-hirudin produced in *E. coli* (Aventis Pharma, Frankfurt, Germany) to demonstrate the compatibility of the established assay.

2.2. Reagents

Sodium hydrogenphosphate (Merck, Darmstadt, Germany), boric acid (Riedel-de Haen, Seelze, Germany) were used as background electrolytes. Tetramethylammonium sulfate, the ion-pairing reagent hexanesulfonic acid, the cationic divalent amine 1,5-diaminopentane (Fluka, Buchs, Switzerland) and the zwitterionic reagent aminoethanesulfonic acid (Merck) were used as modifiers in the migrating solution. Sulfuric acid (Riedel-de Haen) was used for pH adjustment. Sodium hydroxide (Riedel-de Haen) was used to prepare the capillary equilibration solutions. Mesityloxide (Merck) and cholecystokinine flanking peptide (Phoenix Pharmaceuticals, Mountain View, CA, USA) were used as internal migration time markers. Distilled–deionized water was obtained from the Milli-Q laboratory water processing system ELIX 3 (Millipore, Eschborn, Germany).

2.3. Buffer

All solutions were made with Milli-Q (MQ) water and filtered by a 0.2 μm filter before use. Stored at

2–8 °C in the dark, they were stable for several weeks. The stock solutions were as follow: 250 m*M* disodium hydrogenphosphate, 500 m*M* boric acid, 200 m*M* tetramethylammonium sulfate and 1 *M* sulfuric acid.

A 1-g amount of aminoethanesulfonic acid and 1.02 g hexanesulfonic acid sodium salt monohydrate were dissolved in 140 ml. A 40-ml volume of disodium hydrogenphosphate (250 m*M*), 4 ml boric acid (500 m*M*), 2 ml tetramethylammonium sulfate (200 m*M*) and 24 μl diaminopentane were then added. The buffer solution was adjusted to pH 7 with sulfuric acid (1 *M*). Finally, the volume was completed to 200 ml with MQ water which results in the following buffer system: 40 m*M* aminoethanesulfonic acid, 25 m*M* hexanesulfonic acid, 50 m*M* sodium phosphate, 10 m*M* borate, 2 m*M* tetramethylammonium sulfate, 1 m*M* diaminopentane, adjusted to pH 7 (1 *M* sulfuric acid).

2.4. Internal markers

The mixture of the internal markers was prepared as follows: 26 μl MQ water+6 μl CCK solution (1 mg/ml)+8 μl mesityloxide solution (10-fold diluted in MQ water and well shaken directly before addition).

2.5. CE equipment

The instrumentation used for all measurements was an Applied Biosystems (ABI, Foster City, CA, USA) 270A-HT CE system, equipped with a monochromatic UV detector. A fused-silica capillary, 50 μm I.D.×363 μm O.D. (Supelco, Bellefonte, PA, USA) was attached to the system. The detection window was initially created by glowing off the polyimide coating at an effective length of 80 cm using a CE capillary burner (Electrokinetic Technologies, Broxburn, UK). Data were recorded via a Perkin-Elmer (San Jose, CA, USA) 900 series interface. All peak information was obtained through the PE Nelson PC-integrator software, version 5.1 [21].

2.6. Separation conditions

After installation to the system, a brand-new capillary was equilibrated with 2 *M* NaOH for 30 min, with 1 *M* NaOH for 90 min, with 0.1 *M* NaOH for 30 min, and finally with Milli-Q water for another 30 min (application of a vacuum of approx. 650 mbar at outlet end of the capillary). Capillary conditioning between runs was effected by flushing with water, 1 *M* NaOH and 0.1 *M* NaOH for 2 min each, and with running buffer for 6 min to prepare the capillary for the immediate separation. Due to a decline in separation efficiency, after a series of approximately 30 runs the capillary needs to be conditioned as follows: 10 min with 2 *M* NaOH, 30 min with 1 *M* NaOH, 10 min with 0.1 *M* NaOH, and 10 min with water.

After CZE analysis the capillary was flushed with water for 10 min and then flushed from an empty vial for 5 min. The second rinse fills the capillary with air and prepares it for a dry storage without plugging.

Solute detection was effected at a UV absorbance of 200 nm. As a consequence of the small dimensions of the capillary, which enables nl sample volumes, a sample concentration of approximately 5 mg/ml was necessary to visualize structural deviants in the range of 0.10%. Presuming an ideal injection time of approximately 1 s, all samples were introduced on the anode side of the capillary by vacuum injection at 169 mbar. The separation voltage was 20–22 kV (generating a current of 42–48 μA), with the capillary temperature set at 30 °C.

3. Results

New biotechnology has enabled production and purification processes for lepirudin. However, besides the desired lepirudin, related substances including structural deviants and degradation products may be likewise present. The primary structure of lepirudin gives a relative molecular mass of approximately 7000 and an isoelectric point (p*I*) in the range of 4. With this in mind two separation strategies were conceivable, working either at an acidic pH of 3 or at a neutral pH of 7. Working above or below the p*I* value changes the solute charge and causes the solute to migrate either before or after the electroendoosmotic flow (EOF). At a buffer pH of 3 r-hirudin possesses a slightly positive charge, and thus comigrates with the suppressed

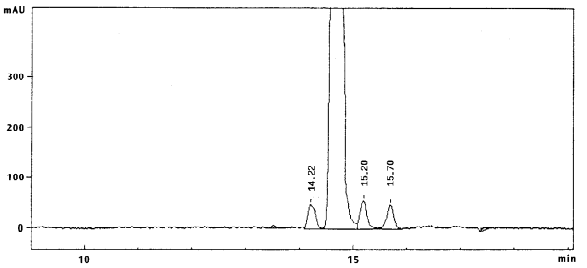

Fig. 2. CZE analysis of r-hirudin (*S. cerevisiae*). Conditions: buffer, 60 m*M* sodium citrate, 40 m*M* sodium borate, pH 3; capillary, 72 cm×50 μm; detection, UV at 190 nm; sample concentration, 10 mg/ml; injection, 1 s vacuum at 169 mbar; oven temperature, 30 °C; current, 25 kV (17 μA).

EOF towards the cathode. This fast separation using citrate and borate as buffer salts, resulted in a large main peak and three baseline separated peaks of smaller size (Fig. 2).

Phosphate buffers may dynamically modify the capillary wall by converting residual silanols on the capillary surface to phosphate complexes which are more easily protonated. Using phosphate as the migrating buffer component, a baseline separation of at least five by-products from the main peak was obtained, but the analysis time was longer (Fig. 3).

At neutral pH the p*I* of r-hirudin is less than the pH of the buffer, thus the electrophoretic mobility of the solute is towards the anode. Buffers of neutral or high pH are generate a relatively high EOF, which prevents the negatively charged solute from migrating from the capillary and carries the solute towards the cathode. The competition between r-hirudin and the oppositely directed EOF, in combination with a

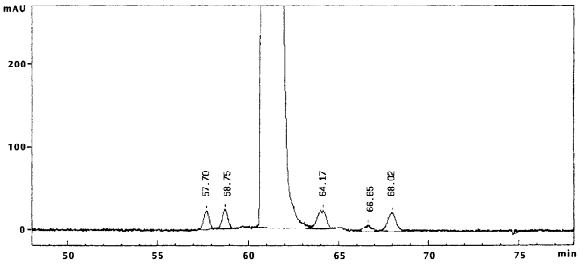

Fig. 3. CZE analysis of r-hirudin (*S. cerevisiae*). Conditions; buffer, 100 m*M* sodium phosphate, pH 3; capillary, 72 cm×50 μm; detection, UV at 190 nm; sample concentration, 10 mg/ml; injection, 1 s vacuum at 169 mbar; oven temperature, 30 °C; current, 15 kV (65 μA).

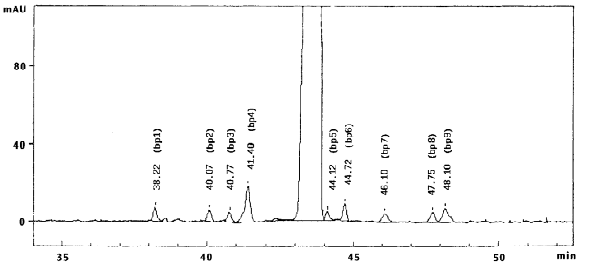

Fig. 4. CZE analysis of r-hirudin (*S. cerevisiae*). Conditions; buffer, 50 m*M* sodium phosphate, 40 m*M* aminoethanesulfonic acid, 25 m*M* hexanesulfonic acid, 10 m*M* sodium borate, 2 m*M* tetramethylammonium sulfate, 1 m*M* diaminopentane, pH 7; capillary, 101 cm×50 μm; detection, UV at 200 nm; sample concentration, 5 mg/ml; injection, 0.7 s vacuum at 169 mbar; oven temperature, 30 °C; current, 22 kV (48 μA).

complex buffer solution, leads to an excellent separation result. In total, nine related substances were baseline separated from the r-hirudin main peak (Fig. 4). All by-product peaks have been identified (Table 1).

Despite the enhanced separation of more components, the use of a long capillary is necessary. Since this contributes to band broadening, however the use of a meandering installed capillary, 102 cm (80 cm effective length)×50 μm in size, generates the desired peak profile. Because of decreasing migration times (MT) and a resultant decline in

Table 1
Determination of [Leu^1,Thr^2]-63-desulfohirudin structural deviants by CZE

CZE peak	Classification (structural alteration)
bp 1	[Leu^1,Thr^2,iso-Asp^{33},imido-Asp^{53}]-63-desulfohirudin and/or [Leu^1,Thr^2,imido-Asp^{33}]-63-desulfohirudin
bp 2	[Leu^1,Thr^2,imido-Asp^{53}]-63-desulfohirudin
bp 3	[Leu^1,Thr^2,mannosyl-Thr^7]-63-desulfohirudin
bp 4	[Leu^1,Thr^2,mannosyl-Thr^4]-63-desulfohirudin
mp	[Leu^1,Thr^2]-63-desulfohirudin (main peak)
bp 5	[Leu^1,Thr^2,des-Gln^{65}]-63-desulfohirudin and/or [Leu^1,Thr^2,phospho-Thr^{45},des-Gln^{65}]-63-desulfohirudin
bp 6	[Leu^1,Thr^2,des-Leu^{64},des-Gln^{65}]-63-desulfohirudin and/or [DesLeu^1,Thr^2]-63-desulfohirudin
bp 7	[Leu^1,Thr^2,iso-Asp^{52}]-63-desulfohirudin
bp 8	[Leu^1,Thr^2,phospho-Thr^{45}]-63-desulfohirudin
bp 9	[Leu^1,Thr^2,desamido-Gln^{65}]-63-desulfohirudin

resolution of the main peak (mp) and by-product peak (bp) 5, the capillary needs to be conditioned after approximately 30 runs. A quotient of the MT of bp 5 through mp should be between 1.005 and 1.015, and functions as a parameter for system suitability testing (SST)—see Table 3. Although a well known electropherogram profile in routine analysis is usually obtained, showing the exact structural determination of the deviants, especially concerning peak identification during the purification process by several spiking experiments, a double-correction method (DCM) of the migration times was established, using mesityloxide (Mes) and cholecystokinine (CCK) as internal markers [22]. Mesityloxide acts as a neutral marker, and therefore comigrates with the EOF, whereas CCK has an acidic p*I* of 2.75, and thus migrates much more slowly than r-hirudin (Fig. 5). The algorithm of the DCM comprises the following two equations:

Correction 1:

$$Rm = (RT_{peak} - RT_{Mes})/(RT_{CCK} - RT_{Mes})$$

Correction 2:

$$Rf_{bp} = Rm_{\text{by-product peak}}/Rm_{\text{main peak}}$$

where RT is the measured migration time, Rm is the relative migration value, and Rf is the corrected migration value. For by-products migrating faster than the main peak, the Rf value is below 1, and for those migrating at a slower rate, the Rf value is more than 1 [23].

Fig. 6 shows the electropherogram of the r-hirudin raw product prior to purification. The resolution power results in a manifold peak profile, and so the method represents an useful tool for in-process control analysis.

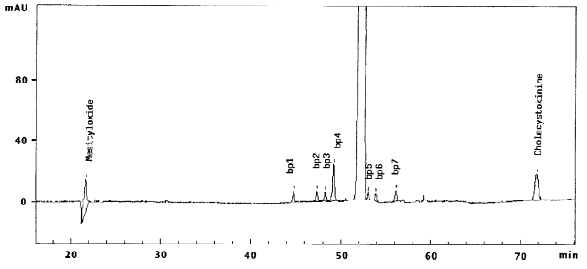

Fig. 5. CZE analysis of r-hirudin (*S. cerevisiae*). Double-correction method via the application of two internal standards. Conditions as in Fig. 4, except current (20 kV).

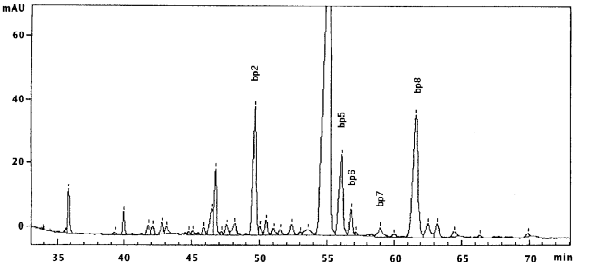

Fig. 6. CZE analysis of lepirudin raw product prior to purification. Conditions as in Fig. 5.

With respect to quality control analysis, the precision of the method in determining the peak area percent values was evaluated by six consecutive runs of a final product sample (Table 2). The relative standard deviation (RSD) for total related protein (TRP) was 2.53. Furthermore, range and linearity of the method were demonstrated (Table 3). A comparable RSD of 3.09 for TRP was achieved. As a result of the purity of the sample, bp 8 and bp 9 were not detected. The robustness was then tested by the analysis of stability samples. Stored in solution at 2–8 °C for 6 months, an increase of up to 2% of bp 7 was obtained. A new peak, bp 7m was also observed (Fig. 7).

Fig. 8 demonstrates the increase of bp 7 within a stability study at 10 °C. In contrast, bp 1 and bp 2 increased their dependence on time and temperature if stored after lyophilization (Fig. 9a, b). The results of a stability study of the lyophilized product at an incubation temperature of 25 °C can be seen in Table 4.

To confirm the reversibility of the formation of bp1 and bp2 (imido-structures) a sample stored under lyophilized condition for 36 months at 25 °C was reconstituted. CZE measurements taken at incubation times of 0, 3, 6, 12, 24 and 48 h in solution at 25 °C showed a decrease of by-products 1 and 2, and a concomitant increase of bp 7 (Fig. 10).

Finally, the compatibility of the method was demonstrated by the application of a sample of r-hirudin produced in another host (*E. coli*). Although the electropherogram profile is slightly different, an excellent baseline separation is also obtained (Fig. 11).

Table 2
Determination of precision by six consecutive runs

CZE run No.	Peak area proportion (%)										
	bp 1	bp 2	bp 3	bp 4	mp	bp 5	bp 6	bp 7	bp 8	bp 9	TRP
1	0.16	0.17	0.15	0.81	98.24	0.13	0.15	0.19	0.00	0.00	1.76
2	0.17	0.18	0.15	0.78	98.15	0.18	0.13	0.22	0.05	0.00	1.85
3	0.16	0.19	0.15	0.79	98.19	0.17	0.13	0.21	0.00	0.00	1.81
4	0.16	0.19	0.15	0.78	98.16	0.17	0.14	0.22	0.04	0.00	1.84
5	0.17	0.17	0.15	0.79	98.16	0.19	0.16	0.22	0.00	0.00	1.84
6	0.18	0.18	0.14	0.80	98.10	0.21	0.17	0.22	0.00	0.00	1.90
Mean	0.17	0.18	0.15	0.79	98.17	0.18	0.15	0.21	0.02	0.00	1.83

TRP = Total related protein.

Table 3
Range and linearity of the method as well as double-correction method data and SST values

RT_{Mes} (min)	RT_{mp} (min)	RT_{CCK} (min)	SST value	Concentration (mg/ml)	Peak area in total	Peak area proportion (%)								
						bp 1	bp 2	bp 3	bp 4	mp	bp 5	bp 6	bp 7	TRP
22.30	54.82	77.08	1.012	2.0	1.760	0.18	0.18	0.16	0.77	98.14	0.17	0.15	0.24	1.86
21.97	54.42	75.82	1.010	3.0	2.578	0.17	0.16	0.14	0.77	98.24	0.14	0.14	0.24	1.76
21.82	53.62	73.87	1.009	4.0	3.444	0.17	0.19	0.16	0.79	98.19	0.13	0.16	0.21	1.81
21.67	52.68	71.90	1.008	4.5	3.699	0.17	0.17	0.15	0.78	98.24	0.13	0.14	0.22	1.76
21.50	51.68	69.75	1.008	5.0	4.209	0.16	0.19	0.15	0.76	98.25	0.13	0.15	0.22	1.75
21.37	50.82	67.90	1.007	5.5	4.547	0.18	0.19	0.15	0.77	98.19	0.13	0.16	0.23	1.81
21.25	50.05	66.43	1.007	6.0	4.707	0.19	0.23	0.16	0.79	98.10	0.15	0.15	0.22	1.90
21.10	49.27	64.72	1.006	7.0	5.255	0.17	0.19	0.15	0.80	98.13	0.17	0.15	0.24	1.87
21.07	48.98	64.00	1.005	8.0	6.015	0.17	0.19	0.15	0.79	98.13	0.18	0.16	0.24	1.87
Mean						0.17	0.19	0.15	0.78	98.18	0.15	0.15	0.23	1.82

As a consequence of sample purity, bp 8 and bp 9 were not detected.

4. Discussion

Whereas the CZE separation of small peptides is relatively straightforward and well understood it appears that no single strategy can be reliably applied to large peptides and proteins. The key element contributing to the success of the present CZE separation is the composition of the buffer. The type of buffer, its ionic strength, the present additives, its viscosity and the properties of the capillary wall affect the efficiency, selectivity and resolution of the separation. The ease with which the selectivity can be changed, is one of the clear advantages of CE over other complimentary techniques. This can be achieved through a number of approaches including the use of buffer additives [24–27].

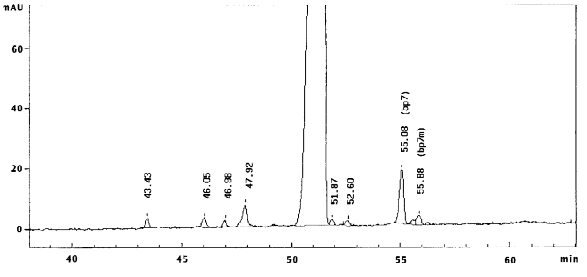

Fig. 7. CZE analysis of lepirudin stability in solution sample (6 months at 2–8 °C). Conditions as in Fig. 5.

Based on this, a complex buffer system has been developed to achieve the present peak profile. Buffers based on borate, phosphate, sulfate or combinations thereof are active participants in different systems. Following on the complexity of the present separation a mixture of sodium phosphate, borate, and tetramethylammonium sulfate constitutes a manifold background electrolyte [28]. Buffer addi-

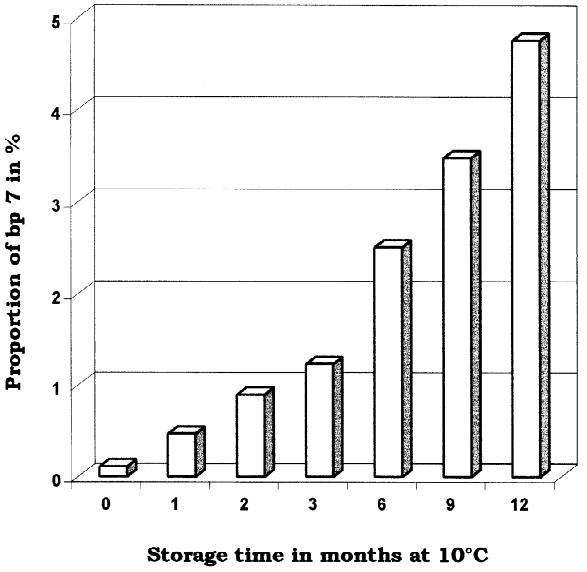

Fig. 8. Stability in solution study of by-product 7 at an incubation temperature of 10 °C.

tives are used to optimize selectivity. The addition of the ion-pairing reagent hexanesulfonic acid to the buffer results in resolution enhancement by hydrophobic pairing between the short alkyl chain of the

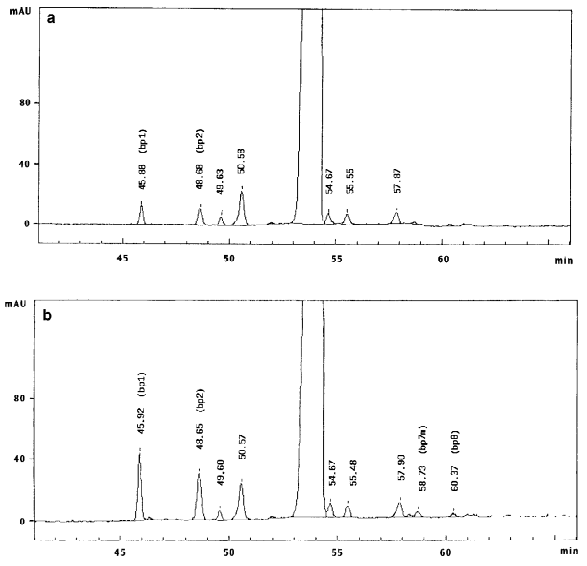

Fig. 9. (a) CZE analysis of a lyophilized lepirudin (Refludan) stability sample (30 months at 2–8 °C). Conditions as in Fig. 5. (b) CZE analysis of a lyophilized lepirudin (Refludan) stability sample (30 months at 25 °C). Conditions as in Fig. 5.

sulfonate and hydrophobic surfaces of the r-hirudin molecules. Zwitterionic buffer reagents such as aminoethanesulfonic acid contain both positive and negative ionizable groups, where a secondary amine group provides the positive charge whilst a sulfonic acid group carries the negative charge [29]. Furthermore zwitterions do not contribute to the conductivity but shield the electrostatic attraction as the positively charged ammonium interacts with the negatively charged silanol groups on the capillary surface [30].

Moreover, the addition of small amounts (1–2 m*M*) of cationic divalent amines, e.g., 1,5-diaminopentane, to the separation buffer results not only in suppression of solute–capillary wall interactions, but also in a decrease of the EOF [31]. At the applied neutral buffer pH r-hirudin and its structural alterations are negatively charged, so they all migrate at a slower rate than the EOF. In this case, the longer the solutes stay on the capillary, the higher their electrophoretic mobility and their charge-to-mass ratio, respectively.

In combination with other microanalytical methods, such as mass spectrometry, sequence analysis, trypsin digestion, amino acid analysis, hydroxylamin cleavage, and HPLC analysis, the established CZE method represented an important tool for identifying the structural alterations of yeast-derived r-hirudin [32]. Some closely related substances differ only in that an isoaspartyl replaces an aspartyl binding. Two aspartic acids (Asp) in positions 33 and 53 are exchanged to asparagine (Asn). Structural differences are mainly caused by deamidation and isomerization of Asn. Geiger and Clarke [33] explained that Asn–Gly and even Asp–Gly structures undergo deamidation. First a succinimide (imido binding) is built, followed by hydrolysis to aspartyl or isoaspartyl. The reversible formation of a succinimide results in a reduction of 18 in molecular mass, and therefore the proportion of these structural deviants increase dependent on time and temperature if stored under dry condition after lyophilization (Fig. 9, Table 4). Furthermore, the presence of a succinimide structure results in one negative charge less. Therefore, these by-products (bp 1, bp 2) migrate faster towards the cathode, followed next by bp 3 and bp 4, which are formed through mannosylation of threonine 7 and 4. By-products 5 and 6, formed by C-terminal trunca-

Table 4
Storage stability of lyophilized lepirudin (Refludan) at 25 °C

Storage period (months)	Peak area proportion (%)								
	bp 1	bp 2	bp 3	bp 4	mp	bp 5	bp 6	bp 7	TRP
0	0.16	0.20	0.17	0.79	98.33	0.15	0.15	0.00	1.67
1	0.25	0.26	0.19	0.89	97.85	0.25	0.18	0.13	2.15
3	0.35	0.35	0.15	0.83	97.82	0.16	0.15	0.07	2.18
6	0.46	0.43	0.19	0.87	97.59	0.20	0.15	0.11	2.41
9	0.54	0.53	0.17	0.86	97.42	0.21	0.14	0.10	2.58
12	0.67	0.64	0.17	0.88	96.99	0.29	0.17	0.15	3.01
18	0.85	0.75	0.16	0.90	96.60	0.25	0.15	0.16	3.40
24	0.95	0.88	0.16	0.89	96.49	0.28	0.18	0.17	3.51
30	1.01	0.92	0.16	0.86	96.38	0.28	0.14	0.11	3.62
36	1.09	0.98	0.19	0.81	96.15	0.25	0.13	0.20	3.85
48	1.21	1.21	0.17	0.90	95.79	0.29	0.17	0.18	4.21

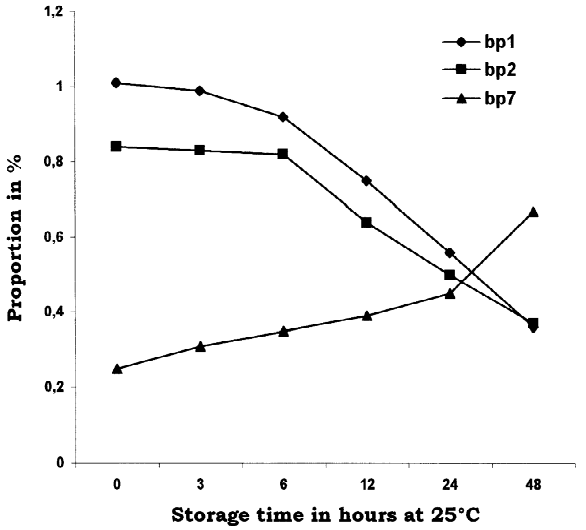

Fig. 10. Storage stability study in solution of by-products 1, 2 and 7 at 25 °C after reconstitution.

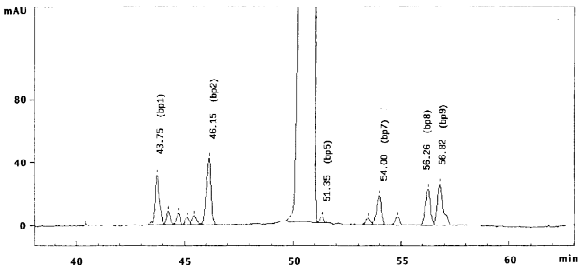

Fig. 11. CZE analysis of r-hirudin (*E. coli*). Conditions as in Fig. 5.

tion mainly by one or two amino acids and N-terminal truncation by one amino acid, migrate first after the main peak. Isoaspartyl (bp 7) should be more acidic because aspartyl can better build intramolecular hydrogen bonds [34,35].

The strongest increase in negative charge occurs through phosphorylation of threonine 45 (bp 8), and deamidation of glutamine 65 (bp 9). These by-products, which could be removed within an intensified fractionation during the purification process yielding lower amounts, are much more negatively charged than the main product, and are thus detected much later than the r-hirudin main peak. During storage in solution, besides an increase in bp 7 (Fig. 8) another by-product which migrates baseline separated on the tide of bp 7, and therefore was named bp 7m, was observed (Fig. 7). Whereas bp 7 was classified as [Leu^{1},Thr^{2},isoAsp^{52}]-63-desulfohirudin, bp 7m was identified as [Leu^{1},Thr^{2},isoAsp^{52}, isoAsp^{53}]-63-desulfohirudin, which results in more acidic behavior, and thus higher charge-to-mass ratio.

In contrast to RP-HPLC, the sensitive CZE method is only suitable for limited quantification, because sample volumes are applied at less than 1 nl (fmol quantities). Nevertheless, this reliable method is highly reproducible because lepirudin and its known structural deviants yield a typical peak profile. After completing the measurement, the result is evaluated by the percentage method in which 100% is given by the sum of all peak areas (but without the peak areas of the two internal markers). In addition, the shift in

migration time is controlled by simultaneous injection of two internal markers and a SST calculation (Table 3). Therefore, the method is a suitable tool for quality control analysis, especially for purity evaluation of final product and stability samples.

The established method is fully applicable to the analysis of r-hirudin, produced in *E. coli*. All the detected by-products are well separated (Fig. 11). In contrast to r-hirudin produced in *S. cerevisiae*, the by-products resulting from mannosylation were not present. In total four small peaks located between bp 1 and bp 2 were obtained in addition. In comparison, phosphorylation and deamidation also occurred, whereas N- and C-terminal truncation was not affected as much as in *S. cerevisiae* production. Therefore, based upon the electropherogram profile the method is also useful for a rapid assignment of the production host.

5. Conclusion

Whenever recombinant proteins are used therapeutically, purity evaluation and characterization of minor impurities are important aspects in quality control. A multiple buffer-additive-strategy enables the HPCE separation of several closely related proteins from the main product. The effective baseline separation, obtained by appropriate combinations of additives, demonstrates the sensitivity of CZE to subtle differences in the charge-to-mass ratio, caused by structural alterations during fermentation, purification or storage stability incubation of r-hirudin. Thus, CZE can play an important role in the complete characterization of biotechnology derived proteins such as r-hirudin. In combination with RP-HPLC, the precise and reliable CZE method can accomplish a separation that is difficult by either technique alone, providing evidence of batch-to-batch consistency of production, as well as performing in process control and stability studies.

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