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Separation of proteolytic enzymes originating from Antarctic krill (*Euphausia superba*) by capillary electrophoresis

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Abstract

Extracts prepared from Antarctic krill (*Euphausia superba*), mainly consisting of acidic proteolytic enzymes, have been studied with capillary electrophoretic techniques. Approximately 50 repeatable peaks were obtained with capillary zone electrophoresis on an untreated fused-silica capillary using a phosphate buffer containing anionic and cationic fluorosurfactant additives as separation medium. A faster separation was achieved on a polyvinyl alcohol coated capillary. Quantitative variations of individual proteins regarding different krill enzyme batches were noted. In the krill samples trypsin-like serine proteinase, carboxypeptidase A and carboxypeptidase B were tentatively identified. © 1998 Elsevier Science B.V.

Keywords: *Euphausia superba*; Antarctic krill; Proteins; Proteolytic enzymes; Fluorosurfactants

1. Introduction

Antarctic krill (*Euphausia superba*) is a crustacean occurring in great amounts in the oceans of the southern hemisphere. Due to its very effective digestion system, krill is able to utilise its food very quickly. The digestion is performed by an array of enzymes, the activities of which are controlled by inhibitors. Post mortem, the inhibitor system is disabled and the krill is rapidly autolysed, due to action of the strong proteolytic enzymes [1,2]. The potential of these enzymes to degrade dead tissue make them attractive for treatment of ulcerative lesions.

Today, there are two main approaches to remove dead or infected tissue from lesions, either by surgery or enzymatic treatment. The enzymatic debriders commercially available do not provide satisfactory results compared to the surgical alternative. However, it has been shown that krill-derived enzymes are significantly more effective in degrading e.g. fibrin compared to trypsin [3,4], one of the most common enzymes used in enzymatic debriders. The cause of this effective degrading is due to the synergistic action of krill enzymes. Eight active enzymes have earlier been identified; three trypsin-like serine proteinases, one chymotrypsin-like serine proteinase, two carboxypeptidase A and two carboxypeptidase B. Throughout this report these enzymes are designated by TL I, II and III, CL, CPA I and II and CPB I and II respectively. The molecular masses of the different enzyme groups are quite similar and have earlier been reported as 33, 29, 28 and 35 kDa [5,6], respectively. The carboxypeptidases have an exopeptidase activity [7], while two of the trypsin-like serine proteinases, TL II and III,

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as well as the chymotrypsin-like serine proteinase have an endopeptidase activity. The third trypsin-like serine proteinase is unique, due to its combined endo- and exopeptidase activities [8,9].

A new debrider based on krill enzymes is currently being developed. The characterisation of these enzymes has hitherto been studied using immunological techniques, liquid chromatography and electrophoresis on polyacrylamide gel [3,5,8,10]. This has provided information about the molecular mass, charge and immunological characteristics of the main components. In connection with ongoing clinical trials of the krill enzyme based debrider, there is a need for additional characterisation of krill extract by high resolution separations. In the present study, capillary electrophoretic methods have been developed. Short analysis times and reproducible patterns, showing the presence of a large number of components, could be obtained. Thus capillary electrophoresis seems to be a suitable technique for quality control of krill enzyme batches.

2. Experimental

2.1. Chemicals and samples

The fluorosurfactants, FC 128 and FC 134, were obtained from 3M Company (St. Paul, MN, USA). Triton X-100 (isooctylfenoxipolyetoxiethanol), was obtained from Kebo (Stockholm, Sweden). Sodium dihydrogen phosphate monohydrate and polyethylene glycol 4000 were obtained from Merck (Darmstadt, Germany). Pharmalyte 2.5-5 was acquired from Pharmacia Biotech (Uppsala, Sweden). Sigma (St. Louis, MO, USA) provided α-lactalbumin. Krill samples were prepared by homogenisation, defatting and gel permeation as previously described [3]. The samples consisting of the 20–40 kDa fraction eluted from a gel permeation column were further purified by affinity chromatography for obtaining the major enzyme components [7,8]. Fractions containing trypsin-like serine proteinase, carboxypeptidase A and carboxypeptidase B were used as reference materials. All original samples and reference materials were lyophilised and stored at −20°C, until use.

2.2. Capillary isoelectric focusing

Capillary isoelectric focusing was accomplished with an eCAP™ cIEF 3-10 Kit on a Beckman P/ACE™ System 5500 with an UV diode-array detector (both items from Beckman Instruments, Fullerton, CA, USA). The ampholyte/sample-gel solution was prepared from 200 μl eCAP™ cIEF gel, 4 μl Pharmalyte 2.5-5 and 2 μl sample solution. To remove bubbles from the solution, it was centrifuged at 4800 rpm for 10 min. The anolyte was prepared from 380 μl eCAP™ cIEF gel and 38 μl 1 *M* phosphoric acid and the catholyte consisted of 20 m*M* sodium hydroxide. The anolyte and catholyte were sonicated for 10 s to remove bubbles. The capillary was rinsed with deionised water for 1 min and then filled with ampholyte/sample-gel solution. Before focusing was started, a 5.4 kV voltage was applied during 3 min to desalt the sample. The focusing was then performed with a voltage of 27 kV during 3 min and the following mobilisation was accomplished with a pressure of 3400 Pa and 27 kV mobilisation voltage. The capillary temperature was 15°C and detection was performed at 280 nm. After separation the capillary was flushed with 10 m*M* phosphoric acid for 2 min and deionised water for 10 min. As p*I* markers, α-lactalbumin, pI 4.8, and CCK flanking peptide, pI 2.75, were used.

2.3. Capillary zone electrophoresis

Capillary zone electrophoresis was performed on a HP ^{3D}Capillary Electrophoresis System with an UV diode-array detector (Hewlett–Packard, Palo Alto, CA, USA). The five different capillaries employed are listed in Table 1 together with operating parameters. All capillaries were of 50 μm ID and of approximately 0.36 mm OD.

For every capillary zone electrophoretic separation a fresh buffer, filtered through a 0.1 μm pore size filter (Millipore, Bedford, MA, USA), was used. The buffer pH was adjusted with HCl or NaOH. The samples and carboxypeptidase B fraction were diluted with deionised water, ten times and one time respectively. The other two reference fractions were used undiluted.

Table 1
Capillaries and experimental conditions employed in CZE

Type of capillary	Untreated fused-silica capillary (1)	Untreated fused-silica capillary (2)	eCAP™ Neutral Capillary	PVA CE Capillary	CElect™-P150 CE Capillary
Capillary source	Hewlett–Packard (Palo Alto, CA, USA)	Hewlett–Packard (Palo Alto, CA, USA)	Beckman (Fullerton, CA, USA)	Hewlett–Packard (Palo Alto, CA, USA)	Supelco (Bellafonte, PA, USA)
Total/effective length [cm]	48.5/40	64.5/56	43/34.5	64.5/56	64.5/56
Injection pressure [Pa]/duration [s]	5000/4	5000/3	5000/3	5000/3	5000/3
Run buffer	25 m*M* Tris, pH 7.0	50 m*M* phosphate, pH 5.5, with various amounts of fluorosurfactant	10 m*M* phosphate, pH 5.5	10 m*M* phosphate, pH 5.5	10 m*M* phosphate, pH 5.5
Capillary temp. [°C]	25	10	10	10	10
Separation voltage [kV]	25	30	−20	−30	−30
Detection [nm]	214	192	192	192	192
Reconditioning	1 min 0.5 *M* NaOH flush, 5 min buffer flush	5 min 0.5 *M* NaOH flush, 60 min buffer flush	5 min buffer flush	1 min 10 m*M* H_3PO_4 flush, 3 min buffer flush	2 min buffer flush

3. Results and discussion

3.1. Capillary isoelectric focusing

Initial capillary isoelectric focusing (CIEF) experiments were performed in a pH 3–10 gradient, but no separation was obtained, while all observed proteins accumulated at the acidic side of the gradient. In subsequent experiments a narrower gradient, pH 2.5–5, was used. From the positions of the markers employed, it could be seen that an acceptable resolution of the krill extract was obtained in the pH range 2.75–4.8, while the resolution in the more acidic region was very poor (Fig. 1). The latter is probably due to protein precipitation, caused by the lack of charge in combination with an instability in the very acidic environment. Neither the addition of an uncharged surfactant, Triton X-100, nor a lowering of the sample concentration solved this problem.

It is known from earlier studies that krill enzymes are very acidic. The isoelectric points of the trypsin-like serine proteinase and carboxypeptidase A have been determined to be 2.6 [11] and 3.6 [12] respectively. It has also been reported that the p*I* of the chymotrypsin-like serine proteinase is below 2.9 [13,14]. Thus, the trypsin-like serine proteinases are probably present in the hump. Carboxypeptidase A is likely to correspond to one or two peaks among the better resolved proteins (13–23 min migration time in Fig. 1), while it is more difficult to predict the location of the chymotrypsin-like serine proteinase.

The problem with the precipitating proteins could perhaps be solved by using buffer additives as described by Conti et al. [15], but no such attempts have been made at this stage.

3.2. Capillary zone electrophoresis on untreated fused-silica capillaries

Zone electrophoretic separations on untreated fused-silica capillaries were initially performed under acidic conditions, but this resulted in a strong protein

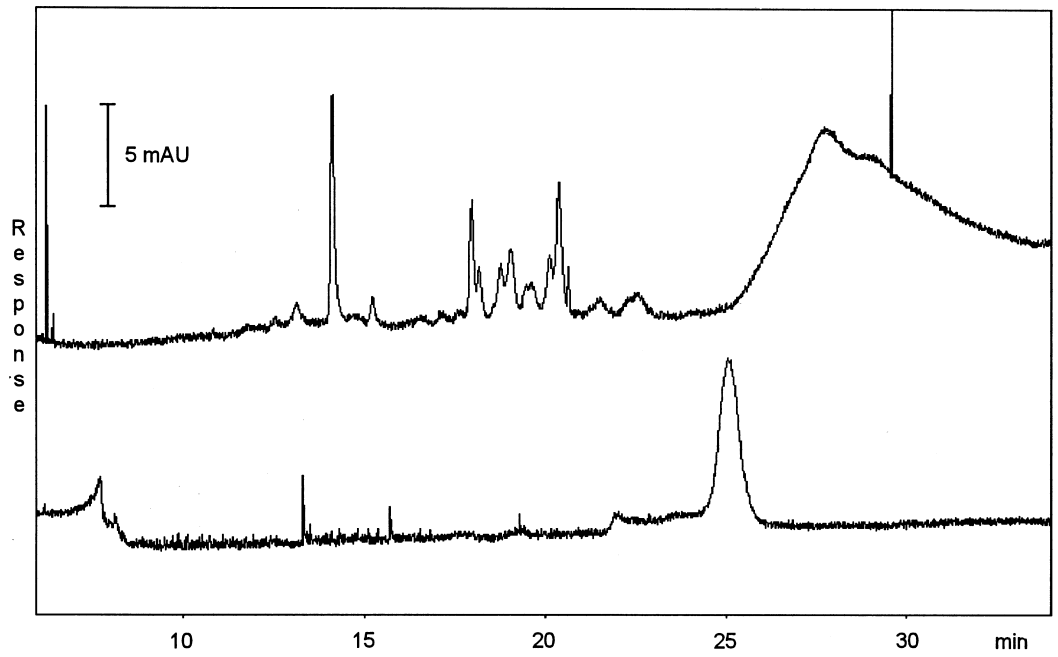

Fig. 1. Upper electropherogram shows the result from a CIEF separation of krill enzyme in 25 m*M* Tris-HCl buffer at pH 7.5 containing 100 m*M* NaCl. After focusing the current was 1.5 μA and at the end of separation the current raised till approximately 5 μA. The lower electropherogram shows reference components, the peak at 8 min corresponds to α-lactalbumin, p*I* 4.8, and the peak at 25 min corresponds to CCK flanking peptide, p*I* 2.75.

adsorption. A better result was obtained with a 25 m*M* Tris-buffer at pH 7.0 (Fig. 2), while buffers with higher pH deteriorated resolution.

Many strategies have been described in the literature to counteract protein adsorption [16]. One of the effective ways of dealing with this problem is the use of fluorosurfactants as buffer additives [17]. Cationic fluorosurfactants adsorb electrostatically to the negatively charged capillary wall and due to hydrophobic interactions between the fluorinated carbon chains, a double layer of surfactants is formed, that exposes a positive charge towards the bulk solution. Thus, the

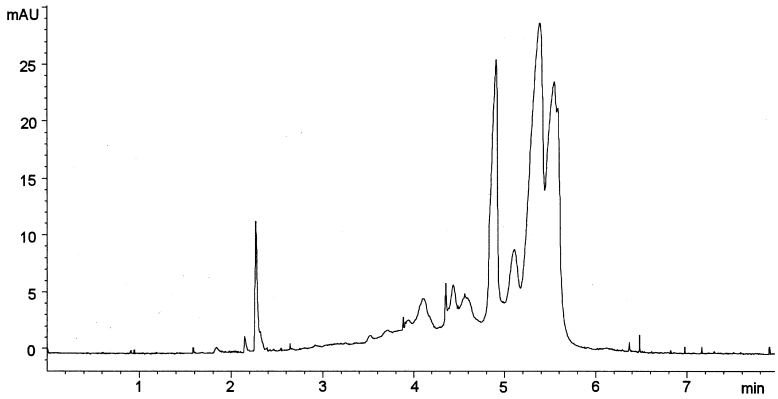

Fig. 2. Capillary zone electrophoresis of krill enzymes, dissolved in the same buffer as in Fig. 1, run on an untreated fused-silica capillary of 48.5 cm total length using 25 m*M* Tris buffer at pH 7.0 as separation medium. The current during separation was ca. 17 μA.

electroosmotic flow (EOF) changes direction. However, this surfactant layer is not suitable for separation of acidic proteins, since these will adhere to the positively charged groups.

By using a mixture of anionic and cationic fluorosurfactants, this problem can be overcome and mixtures of both acidic and basic proteins can be separated [18]. Buffers containing different compositions of the cationic surfactant FC 134 and the anionic surfactant FC 128 were tested. The best results were obtained with a 50 m*M* phosphate buffer with a pH of 5.5, containing 149 μg FC 128 ml^{-1} and 1 μg FC 134 ml^{-1} (Fig. 3). With this buffer composition, which contains a small amount of cationic fluorosurfactant, we assume that the double layer only exposes the anionic fluorosurfactants toward the bulk solution. The EOF is quite strong and the direction is the same as with bare fused-silica. In order to obtain a good repeatability it was necessary to perform a thorough reconditioning of the capillary, which could indicate that there is some residual sample adsorption.

During the experiments with different proportions of cationic and anionic surfactants, it was noted that wall adsorption decreased, as the content of anionic fluorosurfactant was increased, but when the cationic surfactant was omitted, a strong sample adsorption was experienced. Apparently, a small portion of cationic surfactant is necessary to establish and maintain the double layer, which seems to be essential for decreasing sample adsorption.

3.3. Capillary zone electrophoresis on surface modified capillaries

Another strategy to reduce sample adsorption is by permanent deactivation of the capillary wall. Three commercially available surface modified capillaries were tested. Two of the capillaries, the eCAP™ Neutral Capillary and the PVA CE Capillary, have coatings to minimise EOF, while the third, CElect™-P150 CE Capillary have a hydrophilic coating showing a rather strong EOF. Separations on the eCAP capillary (Fig. 4a) were inferior compared to those on the PVA capillary (Fig. 4b). The cause of the impaired resolution of separations on the eCAP capillary was initially expected to be related to the short residence time of the analytes in this column. However, attempts to obtain an improved separation by extending the separation time by means of increasing the viscosity of the buffer through addition of polyethylene glycol did not lead to significant improvements. Separations on the CElect capillary were performed with protein migration and EOF having opposite directions, which also extended the residence time of the proteins. The rather poor resolution obtained with this column (Fig. 4c) is likely to have been caused by interactions between the proteins and the coating or remaining uncoated sites on the capillary wall. Another drawback of this column was that some proteins showed a slower migration rate than the EOF, and therefore migrated from the column on the injection side.

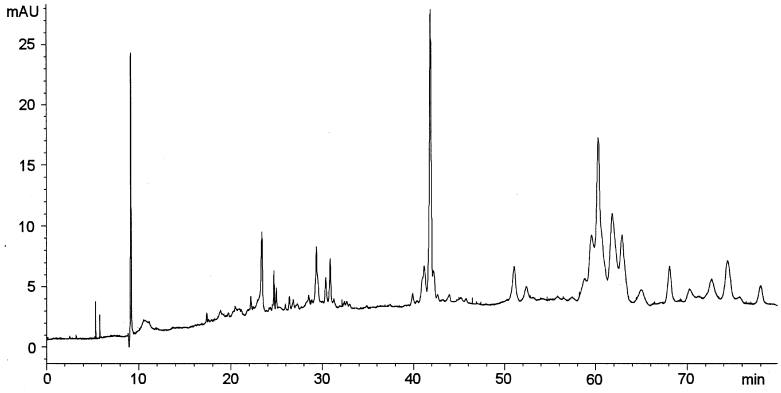

Fig. 3. Capillary zone electrophoresis of krill enzyme, dissolved in the same buffer as in Fig. 1, run on a bare fused-silica capillary of 64.5 cm total length using a 50 m*M* phosphate running buffer, containing 149 μg FC 128 ml^{-1} and 1 μg FC 134 ml^{-1} at pH 5.5. The current was ca. 32 μA.

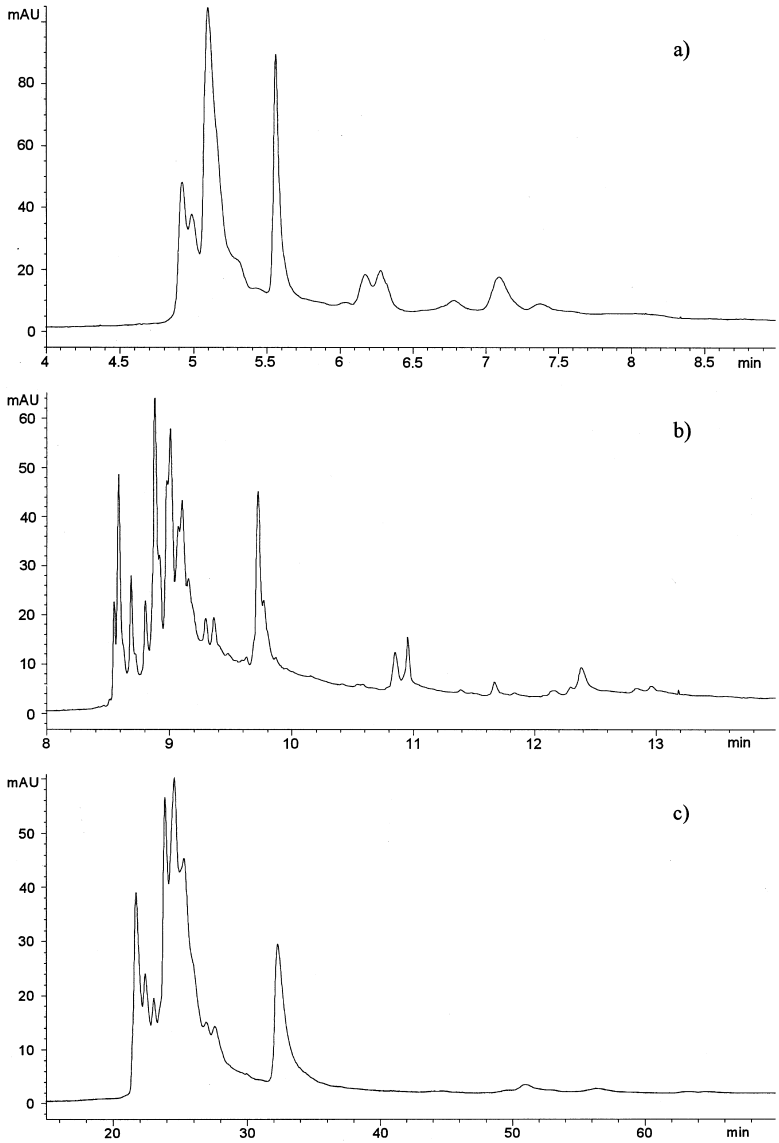

Fig. 4. Capillary zone electrophoresis, using 10 m*M* phosphate running buffer at pH 5.5, of krill enzyme sample, dissolved in the same buffer as in Fig. 1 on (a) eCAP™ Neutral Capillary, (b) PVA CE Capillary and (c) CElect™-P150 CE Capillary. Currents were typically 6 μA.

In order to examine the effect of a lower pH on resolution and protein stability, separations were accomplished on the PVA capillary with a more acidic separation medium than used before. The patterns of the peaks were quite similar in separations performed at pH 5.2 and pH 5.5. At pH 5.0, however, the patterns changed significantly and a deterioration of resolution and repeatability was achieved as well. This could be due to denaturation of the proteins.

3.4. Analysis with reference fractions

In order to obtain a tentative identification of the different groups of enzymes, a comparison with reference samples, previously isolated from the krill

extract by gel permeation and affinity chromatography in combination [7,8], was carried out. The two most successful CE methods; i.e. the one with the untreated fused-silica capillary, employing a phosphate buffer and a mixture of fluorosurfactants, as well as the one employing the PVA coated capillary were used for this purpose. The results obtained are shown in Figs. 5 and 6. The use of fresh buffers showed to be of great importance for obtaining reproducible migration times. However, migration time shifts were still experienced when samples with different ionic strength were separated. In separa-

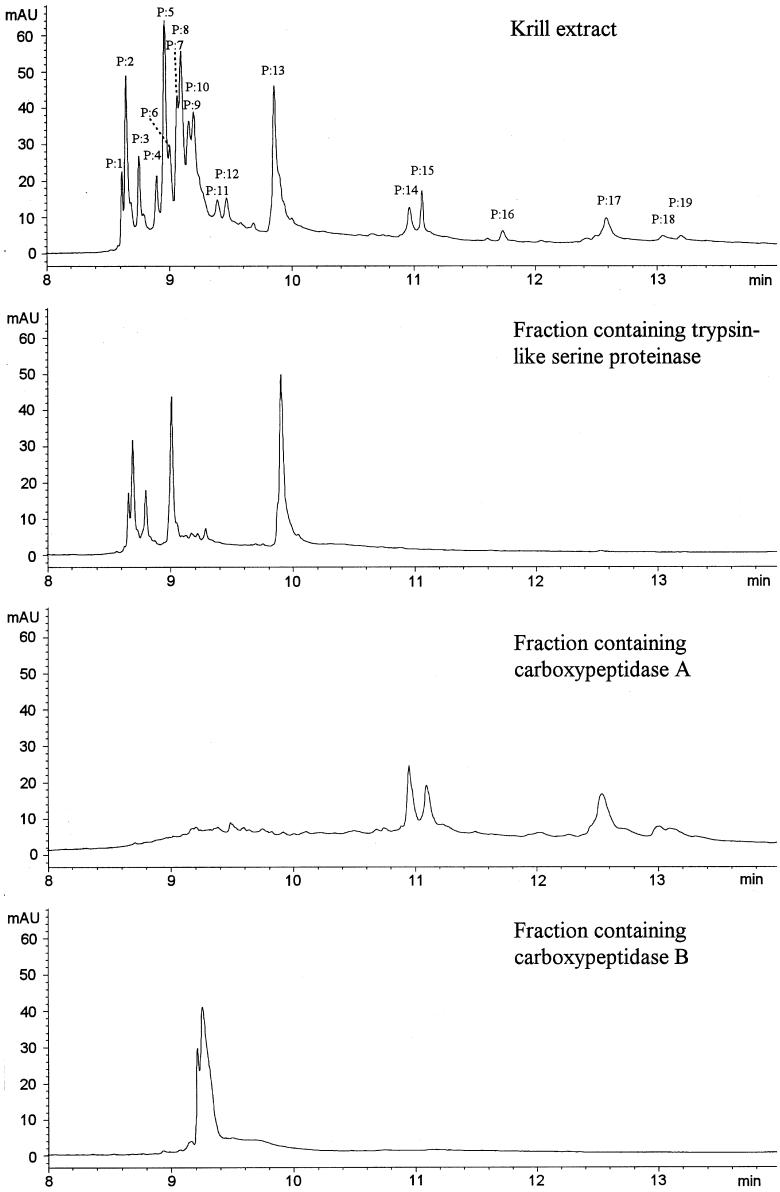

Fig. 5. Capillary zone electrophoresis of krill enzymes and reference fractions, run on a PVA-coated capillary. The krill enzymes are dissolved in the same buffer as in Fig. 1. The samples were stored in a freeze-dried state. After reconstitution in deionised water, the samples contained 50 m*M* Tris-HCl at pH 7.5, except for the carboxypeptidase B fraction of which the buffer concentration was 250 m*M*.

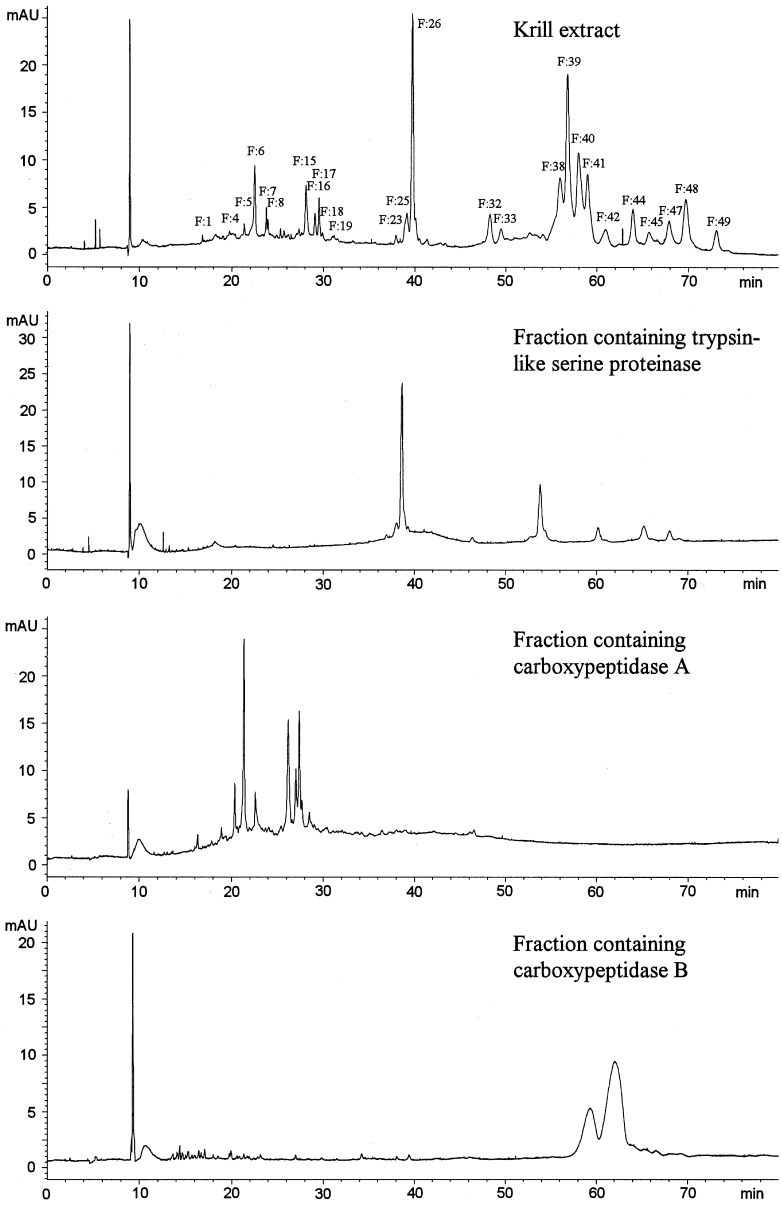

Fig. 6. Capillary zone electrophoresis of krill enzymes and reference fractions, performed on bare fused-silica capillary, using 50 m*M* phosphate running buffer at pH 5.5, containing 149 μg FC 128 ml^{-1} and 1 μg FC 134 ml^{-1}. The same sample and reference fractions were used as in Fig. 5.

tions on the bare fused-silica capillary, fluctuations of migration times were particularly notable.

For the purpose of comparison, peaks are designated *i*:*j* in Figs. 5 and 6, where *i* denotes the method used for the separation and *j* the peak number. Separations with the method employing the PVA coated capillary are marked P and separations carried out with the method using the bare fused-silica capillary are marked F. The peak number, *j*, is obtained by numbering peaks in electropherograms of the krill extract in consecutive order.

In separations on the PVA capillary the trypsin-

like serine proteinase fraction shows five major peaks, that most likely correspond to P:1–3, P:5 and P:13 (Fig. 5). The electropherogram from the separation of the carboxypeptidase A fraction exhibit three main peaks analogous to P:14–15 and P:17 (Fig. 5). In separations of the carboxypeptidase B fraction on the PVA capillary, two peaks, presumably originating from CPB I and II, were detected (Fig. 5). It can be noted that the latter larger peak in this separation is a mixture of components. The carboxypeptidase B fraction had a higher buffer strength than the other reference fractions, which seems to have induced a migration time shift. Identification of these peaks in the krill sample electropherogram is difficult, but their probable locations are among peaks P:7–10.

When comparing the separations, performed with the two CE methods, it can be noted that the order of detection is different, since the electrical polarity is reversed in separations on the PVA capillary. The benefit of the method with the untreated fused-silica capillary is to resolve the least acidic proteins. In separations of the carboxypeptidase A fraction ten peaks, likely to correspond to F:1, F:4–7 and F:15–19 can be observed (Fig. 6). When results from the two methods are compared it can be assumed that peaks F:5, F:6, F:7 and F:8 correspond to peak P:17, while peak F:15 likely is corresponding to P:15 and peaks F:16, F17 and F:18 correspond to peak P:14. Separations of the carboxypeptidase B fraction show two wide symmetrical peaks (Fig. 6). The inferior efficiency of this separation compared to the separations of the other reference materials might be explained by the fact that this fraction has a higher ionic strength.

Similarities could be observed between earlier results achieved by crossed immunoelectrophoresis [5] and results from CE separations on the PVA capillary. Migration rates of the enzyme groups, trypsin-like serine proteinases, carboxypeptidase A enzymes and carboxypeptidase B enzymes are related to each other in a similar way in separations on the PVA capillary and in the first dimension of the immunoelectrophoresis. In both cases the two carboxypeptidase A enzymes migrate with the slowest speed, while the trypsin-like serine proteinases and the carboxypeptidase B enzymes have higher migration rates. Since there is a correspondence between migration rates of the different enzyme groups in the two separation methods, there probably is a correspondence between individual enzymes migration rates as well. Trypsin-like serine proteinases would then be detected in the order TL III, I and II, while carboxypeptidase A enzymes would be detected in the order CPA II and I with the PVA capillary method. Likewise, the detection order would be reversed for separations on the untreated fused-silica capillary.

3.5. Batch variation

Different krill enzyme batches were compared by performing five separations on the PVA capillary on each of four batches (Fig. 7). Migration times were highly reproducible. The relative standard deviation of migration times for three model peaks, P:2, P:13 and P:15, selected from different regions of the electropherogram were 0.03–0.6% within batches and less than one per cent between batches. Also peak heights and patterns were reproducible within batches. The relative standard deviation of the heights of the larger peaks, P:1–10 and P:13 varied within batches between 0.4 and 6.9%. Smaller peaks varied more in height, which might be due to incorrect integration procedures. Small, but distinct differences in peak heights between batches were noted. For example, the peak heights related to the carboxypeptidase A fraction were lower in batch A than in the others. Slight variations in height among the peaks related to the trypsin-like serine proteinase fraction and the carboxypeptidase B fraction could also be seen. Peak P:4, which did not appear in any of the separations of the reference fractions, was one of the major peaks of batch A, while it was almost absent in batch D. Since batch B and C were produced from the same krill raw material, this could explain the similarity between these electropherograms.

4. Conclusions

A novel method has been developed for separation of krill enzymes on an untreated fused-silica capillary, where a buffer containing anionic and cationic fluorosurfactants has been used as separation

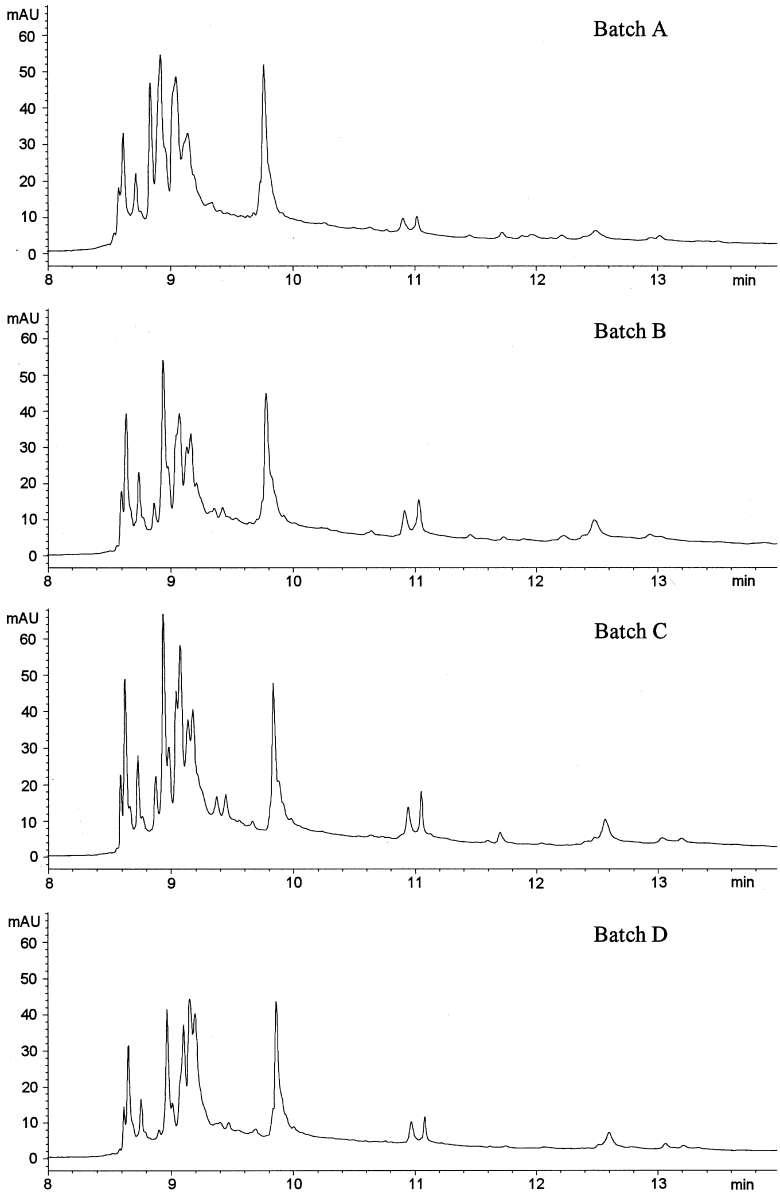

Fig. 7. Capillary zone electrophoresis of four krill enzyme batches run on a PVA CE Capillary. Batch A, B and C were in the same form as the krill enzyme sample in Fig. 1. Batch D was a freeze-dried extract, which after reconstitution in deionised water contained 50 m*M* Tris-HCl buffer at pH 7.5.

medium. With this method, approximately 50 repeatable peaks can be detected in the krill extract. With another method, utilising a polyvinyl alcohol coated capillary, providing a sample throughput of 3 h^{-1}, differences in individual protein concentrations between krill enzyme batches can be detected. The methods seem to be suitable for quality control of a future debrider, based on krill enzymes.

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