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Real-time control of purified product collection during chromatography of recombinant human insulin-like growth factor-I using an on-line assay

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Abstract

During preparative reversed-phase chromatography of recombinant human insulin-like growth factor-I (IGF), the separation of IGF from IGF aggregates cannot be determined using UV absorbance. An on-line reversed-phase chromatographic assay was developed that provides a quantitative measurement of IGF and IGF aggregates every 4 min, allowing real-time control of purified IGF collection. Process control using the on-line assay is a reliable and accurate method to collect purified IGF. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

During chromatography of recombinant proteins, the purified protein product can often be reliably and accurately collected using UV absorbance to distinguish the elution of product and impurities. However, when impurities are not well-resolved from the product, fractions of the column effluent may need to be collected and analyzed to facilitate purified product collection. While fraction collection is efficient for small-scale separations, it is undesirable for large-scale bioprocess chromatography. The fractions must be collected in tanks, using valuable manufacturing plant space. Each fraction must be individually assayed for product purity prior to pooling, which increases quality control costs, increasing the cost of the product. Storing, assaying, and pooling the fractions can significantly decrease product throughput in the manufacturing plant.

When product and impurities are not well resolved, using an on-line assay to quantify the product and the impurities can enable process control by determining when the purified product is eluting. An on-line assay directly samples effluent from the preparative column, and if the assay is sufficiently fast, the purified product can be collected using real-time results from the on-line assay. Methods such as flow-injection immunoassays [1–3], biosen-

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sors [4], and chromatographic assays have been used for on-line analysis. Chromatographic assays have been used for monitoring and controlling fermentation and cell culture processes by measuring product concentration in real-time [5–8], and for monitoring and controlling chromatography processes [9–12]. Chromatographic assays are particularly attractive for on-line use because they are fast and reliable [13–16], and instrumentation specifically designed to run on-line chromatographic assays is commercially available [6].

An example of poorly resolved impurities is the preparative purification of recombinant human insulin-like growth factor-I (IGF) by reversed-phase chromatography. IGF is a 70 amino acid protein [17] with a molecular mass of 7649 and three disulfide bonds [18]. IGF has several important biological functions [19] including growth regulation [20–22], and it may be useful for treating diseases such as Laron syndrome [23,24], diabetes mellitus [25–27], and neurodegenerative diseases [28] including amyotrophic lateral sclerosis [29]. Preparative reversed-phase chromatography is the first chromatographic recovery step in IGF manufacturing. It concentrates IGF and removes most non-protein impurities. It also can separate several variant forms of recombinant IGF, including met^{59}O (methionine sulfoxide at position 59 [30]) and misfolded (where disulfide bonds are improperly formed [31]). However, the primary separation function for this recovery step is to reduce the level of aggregate (aggregated IGF, which includes dimers, trimers and multimers [32]).

This paper describes the preparative purification of IGF by reversed-phase chromatography and discusses the difficulties associated with collecting purified IGF. The development and use of a rapid reversed-phase chromatographic assay that can quantify the amount IGF and aggregate in the preparative column effluent is described. The assay is run on-line for accurate, reliable, real-time control of purified IGF collection.

2. Experimental

2.1. Materials

Bakerbond media was from J.T. Baker (Phillipsburg, NJ, USA). Vydac columns were obtained from Phenomenex (Torrance, CA, USA). Hexylene glycol was NF grade from Ashland (Newark, CA, USA). The BioCAD and BioCAD/RPM chromatography workstations were from PerSeptive Biosystems (Framingham, MA, USA). The HP1090 HPLC system was from Hewlett-Packard (Mountain View, CA, USA). Load material was recombinant IGF produced in bacterial fermentation obtained from Genentech (South San Francisco, CA, USA).

2.2. Analytical chromatography

Off-line analytical chromatography was performed by an high-performance liquid chromatography (HPLC) assay similar to that previously described [33] and previously used for analyzing results from preparative IGF purification [34]. The off-line assay, run on an HP1090, uses a 250 mm×4.6 mm I.D. column packed with Vydac C_{18} 5 μm 300 Å media, a flow-rate of 2 ml/min, a column temperature of 50°C, detection at 214 nm, and an injection of 100 μl (containing about 10 μg of IGF). Buffer A was 0.12% trifluoroacetic acid (TFA) in water and buffer B was 0.1% TFA in acetonitrile. The method was: 27.5–28.5% B/9 min, 28.5–40% B/4 min, 40–90% B/2 min, hold 90% B for 1 min, 27.5% B for 4 min.

The on-line assay, run on a BioCAD/RPM, uses a 30 mm×2.1 mm I.D. column packed with Poros R2/M (20 μm polymeric reversed-phase) media, a flow-rate of 3 ml/min (20 ml/min purge), room temperature, detection at 214 nm, and an injection of 5 μl of the preparative column effluent, sampled directly from the process stream. Buffer A was 0.1% TFA in 10% acetonitrile, buffer B was 0.1% TFA in 27% acetonitrile, and buffer C was 0.1% TFA in 60% acetonitrile. The method was: purge 10 ml buffer A, run 20 column volumes (CVs) buffer A, inject, run 15 CVs buffer A, purge 10 ml buffer B, run 25 CVs buffer B, purge 10 ml buffer C, run 25 CVs buffer C. On the BioCAD instrument, purging takes the column out of the flow path, allowing the increased purge flow-rate to rapidly fill the flow path with buffer. After purging, the flow-rate is decreased and the column is then placed back in line. The BioCAD/RPM software automatically integrates the peaks and displays the result immediately after the assay is finished.

2.3. Preparative chromatography

A scale-down model of the process-scale separation was used. Preparative IGF purification was done on a 25 cm×1 cm I.D. (20 ml) column packed with Bakerbond C_4 40 μm 275 Å media, run on a BioCAD. Buffer A was 50 m*M* acetic acid, 50 m*M* citric acid, pH 3.0 and buffer B was 50 m*M* acetic acid, 20 m*M* citric acid, pH 3.0 with 50% hexylene glycol. The column was run at a temperature of 30°C, maintained by a column oven. A flow-rate of 20 CVs/h was used for the equilibration/load/wash, and a flow-rate of nine CVs/h was used for the gradient/regenerate. The method was: equilibrate three CVs 100% A, load, wash two CVs 100% A, gradient 0–50% B over 10 CVs, regenerate two CVs 100% B. Detection was at 280 nm. Loads for specific experiments are given in the text and in the figure legends.

3. Results and discussion

During the initial purification step in IGF recovery, aggregate should be reduced by approximately 50%, from about 40% in the load to 20% in the purified pool, as measured by the off-line assay (Fig. 1). Recovery yield should be maintained at approximately 80%. Preparative reversed-phase chromatography purifies IGF using a solvent gradient (Fig. 2A). By collecting fractions across the eluted preparative peak and analyzing the fractions using the off-line assay, the elution of IGF and impurities can be measured (Fig. 2B). A pool that would meet the separation goal is shown as a horizontal bar in Fig. 2A. The separation of IGF from aggregate (the main goal of this chromatography step) cannot be seen by UV absorbance. As IGF finishes eluting, aggregate begins eluting, and UV absorbance remains relatively constant. Using a more shallow gradient slope or lower loading produces essentially the same result, primarily because of the heterogeneous nature of the aggregate, with dimers eluting relatively early and multimers eluting later. The separation of monomer from dimer is difficult on the relatively low-performance (40 μm) reversed-phase

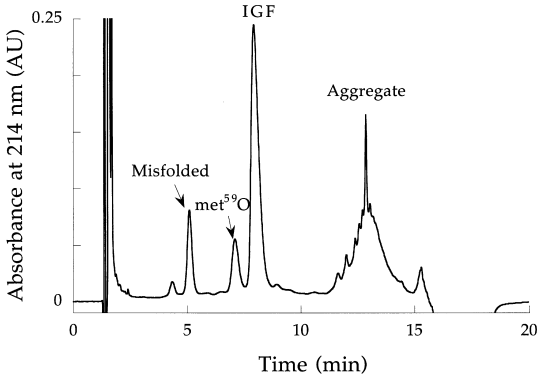

Fig. 1. The off-line assay. The sample assayed here is typical load material for the preparative reversed-phase separation. Aggregate is the sum of peaks integrated from approximately 11.5 min to approximately 14 min.

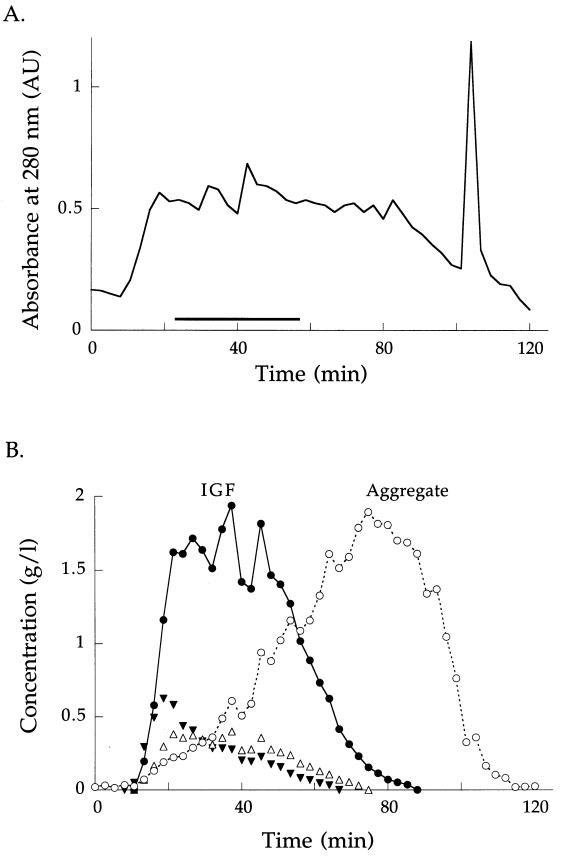

Fig. 2. Elution phase of the preparative purification of IGF by reversed-phase chromatography. (A) UV chromatogram from a typical preparative reversed-phase separation of IGF with load of 12.5 g/l and a gradient of 0–50% B/10 CVs. The solid horizontal line in indicates the purified IGF pool. (B) Analysis of fractions of the preparative peak using the off-line assay. ● IGF, ○ aggregate, △ met^{59}O, ▼ misfolded.

media. Using a very shallow gradient slope and a very low loading can produce a separation that essentially resolves aggregate to baseline. However, using a low load and shallow gradient slope for this bioprocess application would result in an unacceptably low production rate or require an unacceptably large column. Because UV absorbance provides little or no indication of when to begin and end collection of purified IGF, the preparative separation requires a method to reliably and accurately collect purified IGF that does not rely on UV absorbance.

An on-line assay was developed to monitor the elution of IGF and aggregate in real-time. The assay (Fig. 3) is a reversed-phase chromatographic assay with step elution. IGF and the closely related variants met^{59}O and misfolded are eluted as a single peak (labeled ''IGF'' in Fig. 3). Aggregate, which elutes as a wide peak in the off-line assay (Fig. 1), elutes as a single sharp peak in the on-line assay. This peak compression allows reliable integration and peak identification, which is essential for gathering data in real-time. If linear gradient elution was used for the on-line assay, small changes in peak retention time could result in incorrect peak identification, and if peaks were not consistently baseline resolved, unreliable integration could result in incorrect values. Therefore, IGF and aggregate peaks were eluted with step gradients, resulting in well-defined retention times for accurate peak identification, and reliable peak integration for accurate peak measurement.

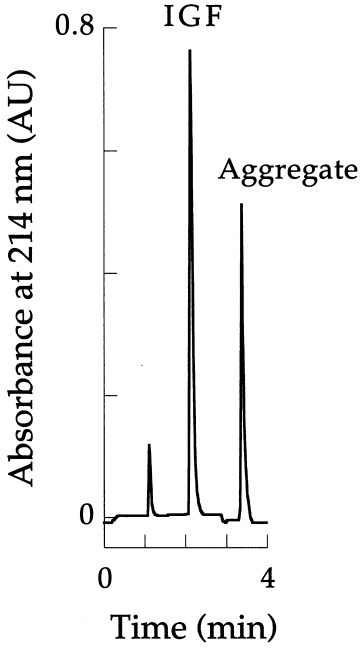

Fig. 3. The on-line assay. With the on-line assay, IGF coelutes with met^{59}O, misfolded, and a portion of the aggregate. The sample assayed here is typical load material for the preparative reversed-phase separation.

The speed of the on-line assay can be increased by several means. The flow-rate can be increased or the volume for washes, elution and equilibration can be slightly decreased. Also, the assay can be run by equilibrating the column in 27% acetonitrile (the amount used for the first step elution), allowing IGF, met^{59}O, and misfolded to flow through and eluting aggregate with 60% acetonitrile. While this method would integrate injection flow-through with IGF, resulting in inaccurate measurement of IGF concentration, it would decrease the assay time to less than 3 min. However, for this application, a 4 min assay time is sufficiently fast to enable real-time control of product pooling. Because the preparative peak is about 120 min wide (Fig. 2), 30 assays can be run over the entire preparative peak, and about 17 assays can be run while IGF is eluting.

The appropriate amount of acetonitrile used in the first elution step of the on-line assay was determined by injecting purified IGF and purified aggregate onto the on-line assay with various amounts of acetonitrile in the first elution step (Fig. 4). As the amount of

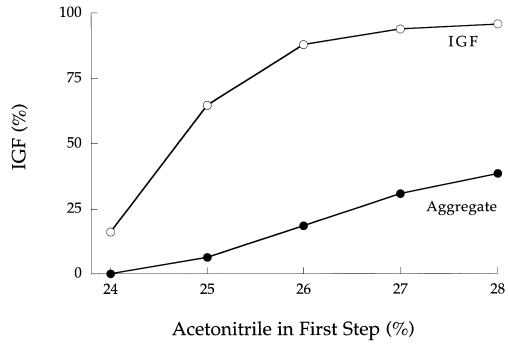

Fig. 4. Development of the on-line assay. Samples of purified IGF (○) and aggregate (●) were injected onto the on-line assay with various levels of acetonitrile in the fist elution step. The amount eluting as IGF (in the first elution step) was calculated as a percent of total peak area.

acetonitrile in the first elution step increases, an increasing amount of aggregate is eluted in the IGF peak, and an increasing amount of IGF is eluted as IGF. Any IGF or aggregate that does not elute in the first elution step elutes in the second elution step (with 60% acetonitrile). Ideally there would be a level of acetonitrile that would completely elute IGF but elute no aggregate, but the step gradient does not permit this. The assay does not accurately measure the absolute amount of IGF and aggregate in the sample (when compared to the off-line assay), but it can be used to accurately control purified product collection.

When sampling the effluent from the preparative peak, the on-line assay produced the same trend as the off-line assay (Fig. 5). Real-time analysis of the preparative peak by the on-line assay shows that IGF elutes first and aggregate elutes next, and aggregate begins eluting before IGF finishes eluting. The preparative IGF peak measured with the on-line assay (Fig. 5) is much larger than the preparative IGF peak measured with the off-line assay (Fig. 2B) because with the on-line assay IGF coelutes with met^{59}O, misfolded, and some aggregate, all of which are well-resolved on the off-line assay. The preparative aggregate peak measured with the on-line assay is smaller than the preparative aggregate peak measured with the off-line assay because a fraction of the aggregate (about 30% by Fig. 4) is eluting with IGF on the on-line assay. By choosing appropriate pooling parameters, the on-line assay can be used to accurately pool purified IGF.

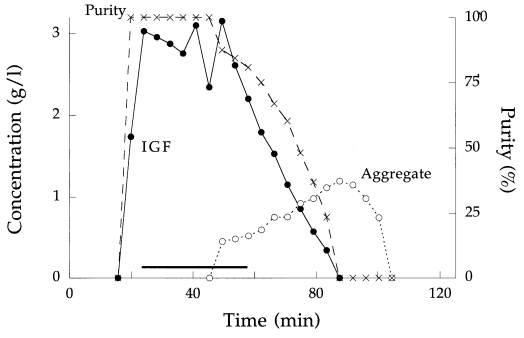

Fig. 5. Real-time results from the on-line assay, sampling the preparative column effluent and providing a quantitative measurement of IGF and aggregate every 4 min. Results are from real-time integration of peaks from the on-line assay used to monitor the preparative process in Fig. 2 (12.5 g/l load and a gradient of 0–50% B/10 CVs). Points are displayed for the time of sampling; the actual results are available about 3 min later. ● IGF, ○ aggregate, × purity, determined by the on-line assay. The solid horizontal line indicates the purified IGF pool.

Collection of purified IGF should begin just after IGF begins eluting, which allows a slight reduction in met^{59}O and misfolded while not significantly decreasing IGF yield. Collection of purified IGF should end as the IGF peak is coming down and the aggregate peak is going up. The on-line assay can be used to collect product using different assay values as a parameter for controlling pooling. Pooling could begin when the concentration or purity of IGF reaches a predetermined value. Pooling could end when the IGF concentration or purity passes below a certain value, or when the aggregate concentration goes above a certain value.

Peak collection using the on-line assay relied on IGF purity, which is the IGF peak area divided by the peak area of IGF and aggregate. Product collection began when IGF purity went above 85% and product collection ended when IGF purity went below 85%. A pool collected from 85% IGF purity to 85% IGF purity measured by the on-line assay (Fig. 5) meets the pool requirements of 20% aggregate and 80% yield. With the on-line assay, samples from the preparative column effluent with less than 0.25 g/l of IGF or aggregate have no peaks integrated because a large peak area reject was used in the integration parameters for the on-line assay. The peak area reject ensures that when small amounts of IGF and aggregate are eluting (which occurs at the very beginning of IGF elution), pooling will not begin. Another, equally robust, method for ensuing this is to set an additional parameter of IGF concentration above 0.25 g/l before pooling begins.

To demonstrate the reliability of using the on-line assay for purified IGF collection, five sequential runs were performed using a IGF load of 12.5 g/l (g IGF per l column volume) and a gradient of 0–50% B over 15 CVs. Purified IGF was collected from 85% IGF purity to 85% IGF purity using real-time results from the on-line assay, and the purified IGF pool was analyzed by the off-line assay. Because the off-line assay is more accurate than the on-line assay, the final purity and yield of the collected product is determined by the off-line assay. The purity of IGF increased from 38.4% in the load to

53.2% (S.D. 0.4%) in the purified pool, including a reduction of met^{59}O and misfolded. The amount of aggregate decreased, from 43.0% in the load to 18.1% (S.D. 0.7%) in the purified pool. Recovery yield was 79.8% (S.D. 2.0%). The low standard deviations observed over the five cycles show that the on-line assay can be used to reliably collect purified IGF. The collected product met the goal of less than 20% aggregate while maintaining recovery yield of 80%.

If the preparative separation was run under identical conditions each time, the pool could be collected by time or volume. However, conditions often change in bioprocess applications from batch to batch. For example, because of batch-to-batch fluctuations in expression levels during fermentation, the load onto the column may change. The on-line assay can reliably and accurately collect a purified pool when the load changes (Fig. 6). The amount of aggregate in the pool and recovery yield remain constant while the pool volume changes significantly. If purified IGF was collected using time or volume, the amount of IGF and aggregate, as well as recovery yield, would fluctuate. This also demonstrates that collecting IGF based on IGF purity is a robust collection method. Pooling begins at 85% purity, just after IGF begins eluting; with a 3 min delay for the assay to run, some met^{59}O and misfolded are removed. Ending collection at 85% purity ensures that collection ends as IGF is ending elution and aggregate is beginning elution. A pooling method based on IGF or aggregate concentration would not have produced equivalent results.

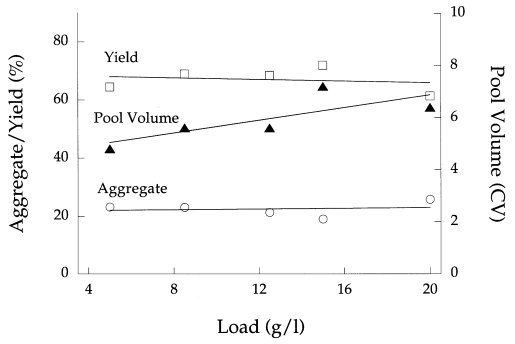

Fig. 6. Results of pools collected from 85% to 85% purity determined in real-time by the on- line assay. The load was 5, 8.5, 12.5, 15 and 20 g/l, and the gradient was 0–50% B over 10 CVs. Collected pools were analyzed by the off-line assay (□ yield, ○ aggregate). Yield and aggregate remain almost constant while the pool volume (▲) is changing. Pool volume was measured as column volumes (CVs).

The on-line assay was able to provide real-time data to reliably and accurately collect purified IGF during preparative reversed-phase chromatography. Although the on-line assay itself does not accurately measure the concentration of IGF and aggregate when compared to the off-line assay, the information it provides is accurate enough to enable real-time control of purified IGF collection. By using step gradients and compressing peaks, reliable integration and peak identification is ensured. By beginning and ending product pooling based on IGF purity measured by the on-line assay, the pooling method is accurate when the column load is changing. The assay was able to provide reliable product pooling over multiple cycles.

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