Bio-Works

APPLICATION NOTE

Purification of peptides by cation exchange chromatography

Therapeutic peptides hold an important position amongst pharmaceutical compounds due to their relatively small size and their tolerability in humans, but they are subject to highly stringent purity requirements. The target peptide can be synthesized, for example by solid phase synthesis, or expressed in a biological model system, both of which generate a crude peptide contaminated by extensive amounts of process-related impurities (failure sequences, chemical modifications, bioburden etc.), that need to be removed. Currently, reverse phase chromatography (RPC) is the most commonly employed purification technique, but crude feeds may foul the columns. By introducing a cation exchange chromatography (CIEX) step upstream of the high-performance silica-based RPC step, the burden from impurities is significantly reduced. In addition, the purity of the target peptide is greatly enhanced. We present here two differently produced therapeutic peptides, both purified with an upstream CIEX step before one or two RPC steps. This purification process provided the desired target peptide purity of more than 99%.

Peptides

Today there are many therapeutic peptides on the market for clinical use. The global market for therapeutic peptides in 2017 amounted to $22 billion and is estimated to exceed $50 billion by 2024. Thus, there is a high demand for robust and high-performance peptide purification strategies.

So-called therapeutic peptides (2-50 amino acid sequences) are attractive as medical candidates due to their high efficacy, safety and tolerability in humans, as well as the lower complexity and cost of production compared to protein-based drugs. Therapeutic peptides represent a specialized niche between protein-based biopharmaceuticals and traditional small molecule therapies and constitute a promising treatment approach to many diseases including cancer. Regardless of the peptide production method (peptide synthesis or isolation from expression in host cells), all crude peptides need to be purified and the purity requirements for therapeutic peptides are particularly stringent.

Peptide purification

Purification of biomolecules from complex feeds, *i.e.*, crude recombinant peptides, is often best carried out using combinations of orthogonal separation methods.

The different peptide production methods pose various challenges regarding process-related impurities. Crude synthetic peptide feeds (solid phase synthesis or liquid phase synthesis) contain impurities such as truncations, deletions and potentially chemically modified sequences. Custom-made peptides are routinely synthesized to up to 50-60 amino acids, but the longer the peptide sequence, the more abortive and erroneous sequences that must be removed in downstream purification processes.

Crude peptides from a cell-based expression system, *e.g.*, *Escherichia coli*, contain many host related impurities, such as lipids, nucleic acids, endotoxins, etc., that are still present after initial sample pretreatment steps. As mentioned before, it is vital that the final peptide product has the required purity to be used as a therapeutic which places high demands on the purification processes.

RPC *vs.* IEX

Reverse phase chromatography (RPC) is the most commonly and frequently used peptide separation technique. As for all purification strategies, this technique has its own challenges and disadvantages. High resolution RPC is expensive and based on silica.

Impurities from the peptide feed are prone to cause fouling of the column that is difficult to remove since the option of cleaning-in-place (CIP) using sodium hydroxide is limited

for silica resins. This therefore results in shortened lifetime of RPC resins. To circumvent these drawbacks for silica resins and to improve the peptide separation, we recommended including ion exchange chromatography (IEX) as a capture step upstream of the RPC step. The peptides can also be more efficiently separated using alternative modes of selectivity, and a variety of peptide modifications that cannot be removed by RPC can be separated by IEX (*e.g.*, deamidation and acetylation).

IEX is dependent on the ionic interaction between the immobilized ion exchange groups on the chromatography resin and charged groups of the peptide. Both cation and anion exchangers have been used with success for peptide purifications. CIEX is more common than AIEX, but it ultimately depends on the peptide sequence.

WorkBeads 40S

WorkBeads™ 40S is an agarose-based strong cation exchange chromatography resin (it is completely ionized over a broad pH range, 2–12) derivatized with sulfonate ligands. This high-capacity resin is manufactured using a proprietary method that results in porous beads with a tight size distribution and exceptional mechanical stability and is stable in high concentrations of sodium hydroxide allowing efficient cleaning-in-place (see Table 1). WorkBeads 40S is thus an excellent choice as an upstream purification step for peptides if a cation exchange chromatography resin is preferred prior to the RPC step(s).

Table 1. Properties of WorkBeads 40S.

WorkBeads 40S	
Average particle size (D_{V50})[1]	45 µm
Ionic group (ligand)	Sulfonate (-SO_3^-)
Ionic capacity	180–250 µmol H^+/mL resin
Dynamic binding capacity (DBC)[2]	130 mg BSA/mL resin
Max flow rate (20 cm bed height 5 bar)	600 cm/h
pH stability	2–13

[1] The median particle size of the cumulative volume distribution.
[2] Dynamic binding capacity determined at 4-minutes residence time in 20 mM sodium citrate, pH 4.0.

Purpose of study

We have studied the advantage of using a CIEX capture step upstream of the RPC step to enhance the peptide purity and at the same time to protect the sensitive downstream RPC resin.

Two different cases have been studied; one with a synthesized crude peptide feed and one with a crude recombinant peptide feed (human insulin precursor).

Case 1: Synthetic peptide purification

We have investigated the capture of a synthetic pharmaceutical peptide of 45-amino acid residues (pI 4.3-4.5) using WorkBeads 40S. The capture step increases the purity of the final peptide product and additionally reduces impurities loaded onto the downstream high-performance silica-based RPC column. Capto™ SP ImpRes (Cytiva) resin was included in the study for comparison.

The binding and elution conditions were optimized for the CIEX capture step, with binding at pH 4 and elution at pH 6 in the presence of 15% acetonitrile. The final peptide load was approximately 20–25% of the dynamic binding capacity (DBC) of the resin to visualize individual separated peptide species.

To validate the results of including WorkBeads 40S as a capture column prior to the RPC column, the results of experiments were analyzed for purity in sub-sequent steps.

Dynamic binding capacity

The dynamic binding capacity was determined by frontal analysis at different flow rates using pure peptide (10 g/L) applied under optimized binding conditions and determined at 10% breakthrough. Figure 1 shows the breakthrough curve for this peptide feed on WorkBeads 40S.

Resin: WorkBeads 40S
Column: 10 × 57 mm, 4.5 mL
Flow rate: 150 cm/h (2 mL/min)
Feed: 10 g pure peptide/L
Binding condition: Acidic with acetonitrile
Elution: Step elution with pH gradient

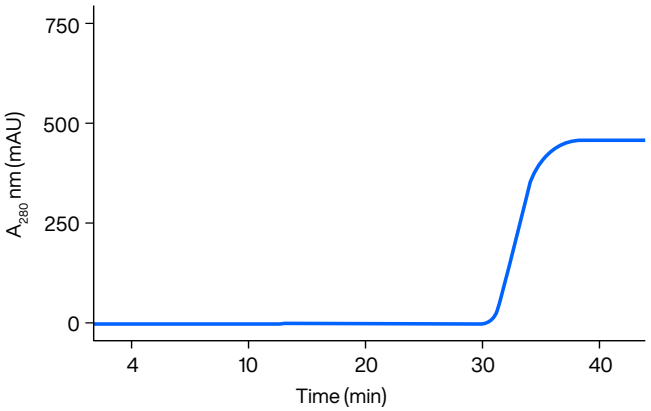

Figure 1. DBC measurements. Breakthrough curve for WorkBeads 40S at a flow rate of 150 cm/h.

DBC for WorkBeads 40S was estimated to 151 mg/mL at 150 cm/h (2 minutes residence time), indicating excellent mass transport. This value was slightly decreased to 140 mg/mL at 300 cm/h (1.1 minutes residence time, see Figure 2).

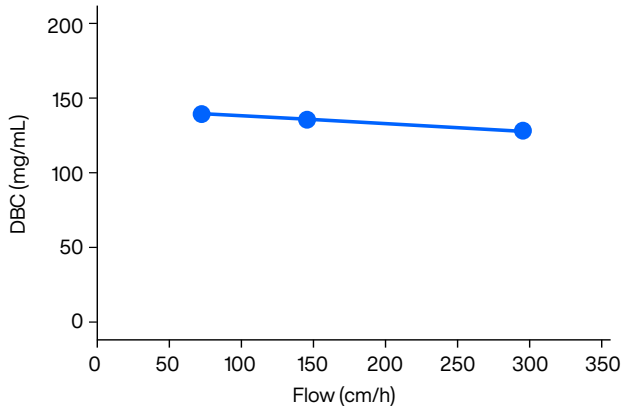

Figure 2. DBC *vs.* linear flow determined for WorkBeads 40S.

DBC for Capto SP ImpRes was determined to 125 mg/mL at 2 minutes residence time and 123 mg/mL at 1.1 minutes residence time, *i.e.*, lower than for WorkBeads 40S (see Table 2).

Table 2. Dynamic binding capacity at different residence times

Resin	DBC (mg/mL) 2.0 min residence time	DBC (mg/mL) 1.1 min residence time
WorkBeads 40S	150	140
Capto SP ImpRes	125	123

CIEX purification of synthetic peptide

A 55% pure peptide feed was purified with WorkBeads 40S and Capto SP ImpRes to establish which of the resins give the most optimal combination of purity and yield. Pre-purification steps had been conducted, such as precipitation, filtration etc., to obtain this starting material, a 55% pure feed. As stated above, approximately 20–25% of the CIEX resins' DBC was loaded to be able to distinguish the separation of impurities from the pure peptide, though, at process scale a higher percentage of the resins' DBC can be loaded to maximize each purification run. See Figure 3, for the separation obtained on the two different CIEX resins.

The main target peptide peak indicated in the chromatogram above is more separated for WorkBeads 40S than for Capto SP ImpRes. All peptide species eluted earlier in the chromatogram are incomplete failure peptide sequences, *i.e.* impurities that must be removed. The target peptide was collected to keep the yield the same (92%) on both columns. The purity obtained with a loading of 30 g/L was 91.8% for WorkBeads 40S and 85.2% for Capto SP ImpRes (Table 3). The purity increased to 93.0% on WorkBeads 40S when the loading was decreased to 10 g/L resin.

Resins:	WorkBeads 40S Capto SP ImpRes
Column:	10 × 240 mm, 19 mL
Flow rate:	150 cm/h (2 mL/min)
Feed:	Crude feed with 30 g peptide/L resin (55% purity of target peptide)
Binding condition:	Acidic with acetonitrile
Elution buffer:	pH gradient with acetonitrile
Gradients	1. Linear: 0% to 40%, 1 CV (column volume) 2. Step: 40%, 2 CV 3. linear: 40% to 70%, 5 CV

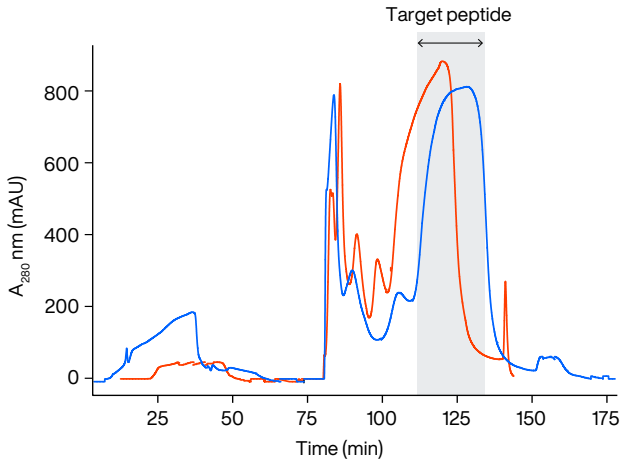

Figure 3. Purification of the 45-amino acid residue peptide from the 55% pure feed. UV traces are shown for WorkBeads 40S (blue) and Capto SP ImpRes (red). Collected pool of target peptide is shown with the grey area and double headed arrow.

Table 3. Purity obtained with a load of 30 g/L of a crude feed containing 55% target peptide.

Resin	Purity (%)	Impurities (%)
Starting feed	55	45
WorkBeads 40S	91.8	8.2
Capto SP ImpRes	85.2	14.8

The peptide pool eluted from WorkBeads 40S was then subjected to a polishing step using an RPC silica column resulting in the final purity of 99 % for this therapeutic peptide (Figure 4).

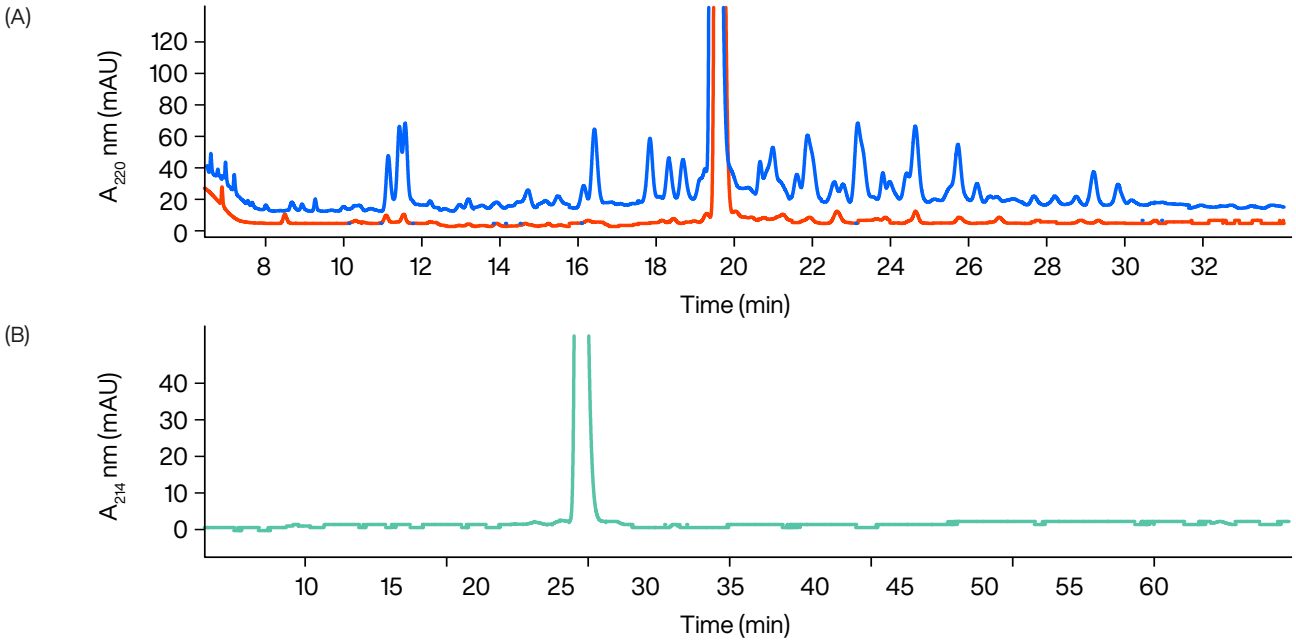

Figure 4. Purity analysis by RPC. (A) Crude feed (blue trace) and peptide purified on WorkBeads 40S (red trace). (B) Final product after RPC polishing.

Summary for synthetic peptide purification

WorkBeads 40S shows superior performance regarding capacity and selectivity (purity/yield) compared to Capto SP ImpRes. Both resins show excellent mass transfer properties and no hindered diffusion.

The added CIEX step using WorkBeads 40S reduced the loaded impurities on the RPC column from 45% to 8.2% (see Table 3) and a final purity of 99% for the target peptide is reached after the combination of CIEX (WorkBeads 40S) and RPC, which is the requirement for pharmaceutical peptides/active pharmaceutical ingrediencies (API). Thus, WorkBeads 40S is an excellent choice to include upstream in the purification flow for synthetic peptide purifications, both regarding final purity of the target peptide and for protection of the downstream silica-based RPC resin.

Case 2: Recombinant insulin purification

We have conducted a study with recombinant human insulin precursor (pI 4.5) expressed in *Escherichia coli,* in which a CIEX capture is the first step prior to RPC purifications. A standard purification process for this recombinant human insulin precursor involves two RPC steps using different buffer systems with different pH for complementary selectivity. When we included an upstream CIEX step we improved the selectivity even further.

The binding and elution conditions were optimized for the CIEX capture step, with buffer at pH 4 in the presence of 30% ethanol. Elution was performed with a salt gradient and the sample volume was approximately 14-16% of the DBC.

A comparison between WorkBeads 40S and Capto SP ImpRes as the first step was also performed.

CIEX purification of insulin precursor

A sample feed containing 72.5% pure human insulin precursor (Met-Lys-Human insulin), was purified in a three-step process. Each purification step was optimized, and fractions were pooled to give a yield of 90% with highest possible purity.

CIEX was used in the first purification step, where the peptide feed was applied to a column packed with WorkBeads 40S. Ethanol was included as an organic modifier. See Figure 5 for the separation of human insulin precursor from impurities.

Resin:	WorkBeads 40S
Column:	10 × 240 mm, 19 mL
Flow rate:	150 cm/h (2 mL/min)
Feed:	20 g insulin precursor feed/L resin
Binding buffer:	50 mM sodium acetate, pH 4 with 30% ethanol
Elution buffer:	50 mM sodium acetate, 1 M NaCl, pH 4 with 30% ethanol
Gradients:	1. Linear: 0% to 20%, 2 CV 2. Step: 20%, 1 CV 3. Linear: 20% to 70%, 6.5 CV

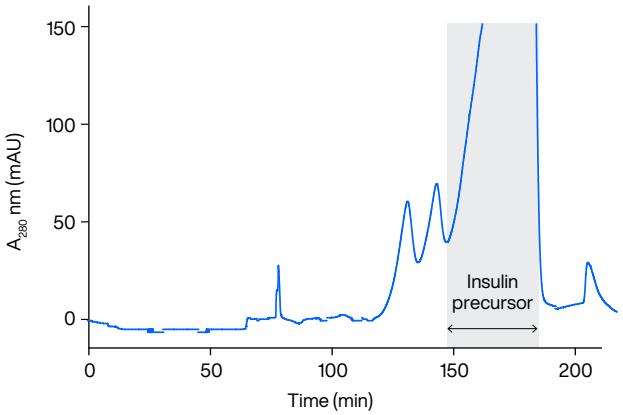

Figure 5. First chromatography step, CIEX, where the insulin precursor feed is separated on WorkBeads 40S. Collected pool of target peptide is shown with the grey area and double headed arrow.

The main peak is collected to obtain a yield of 90% and later further processed. See Table 4 for the purity of the pools collected from the WorkBeads 40S and Capto SP ImpRes, respectively. The CIEX step using WorkBeads 40S reduced the impurities from 27.5% to 12% (Table 4, column 3).

Table 4. Purity comparison between WorkBeads 40S and Capto SP ImpRes for a pool with a yield of 90%.

Eluted pool	Purity (%)	Impurities (%)
Starting feed	72.5	27.5
WorkBeads 40S	88.0	12
Capto SP ImpRes	85.0	15

As shown in Table 4, a slightly higher purity in the first step, can be obtained by using WorkBeads 40S, compared to Capto SP ImpRes (88% *vs.* 85% for a pool yield of 90%).

Three-step purification of insulin precursor

The pool from the CIEX run (WorkBeads 40S) was loaded on the RPC columns for further processing, resulting in a final purity of 99.7%. The second and third purification steps were run with the same RPC column (PK-C8-10µm-100Å), while the running conditions were different for complementary selectivity. The setup for the three-step purification process is shown in Table 5.

Table 6 shows the purity after each step in the process.

Analytical chromatograms of each step in the three-step purification process are shown in Figure 6, where the step-wise enhancement of purity is clearly visualized.

Table 5. Three-step purification process of a recombinant human insulin precursor

	Step 1: IEX	Step 2: RPC	Step 3: RPC
Resin	WorkBeads 40S (Bio-Works, Sweden)	PK-C8-10µm-100Å (Osaka Soda, Japan)	PK-C8-10µm-100Å (Osaka Soda, Japan)
Bed height	24 cm	25 cm	25 cm
Feed	Crude human insulin precursor	Pooled elution fraction from step 1	Pooled elution fraction from step 2
Loading	20 g product/L resin	15 g product/L resin	15 g product/L resin
Binding buffer (A)	50 mM NaAc, pH 4.0: EtOH (7:3)	100 mM $(NH_4)_2SO_4/H_2SO_4$, pH 3.2	200 mM NH_4Ac/HOAc pH 5.5
Elution buffer (B)	50 mM NaAc, 1 M NaCl, pH 4.0: EtOH (7:3)	Acetonitrile	Acetonitrile
Gradient	0 – 20% B in 2 column volumes (CV), 20% B in 1 CV, 20 – 70% B in 6.5 CV, 100% B in 20 CV	5 – 21% B in 1 CV, 21 – 27% B in 6 CV, 60% B in 2 CV	5 – 25% B in 1 CV, 25 – 28% B in 6 CV, 60% B in 2 CV
Flow rate	150 cm/h	180 cm/h	180 cm/h

Table 6. Purity obtained during the optimized three-step purification process of recombinant human insulin precursor.

Step	Purity (%)
Starting feed	72.5
Step 1: WorkBeads 40S	88.0
Step 2: PK-C8-10µm-100Å (condition 1)	97.0
Step 3: PK-C8-10µm-100Å (condition 2)	99.7

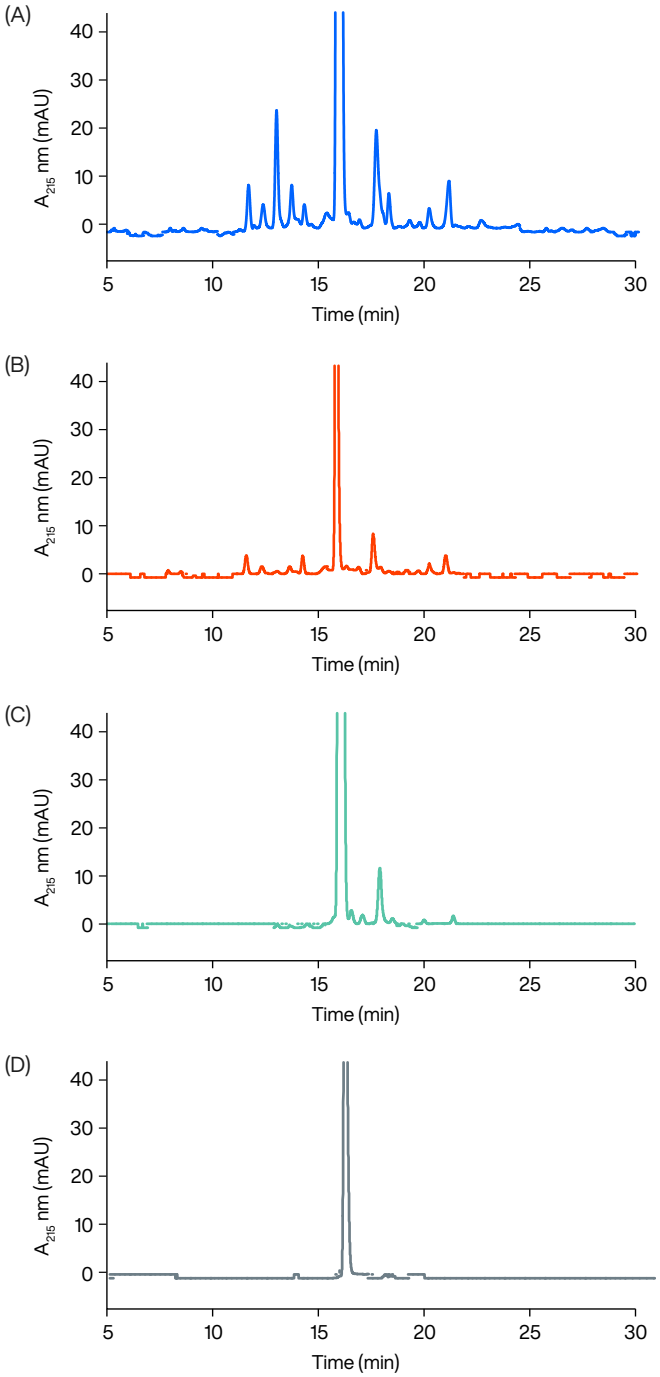

Figure 6. RPC analyses of purified pools from the optimized three-step purification. Feed (A), step 1: eluted pool from WorkBeads 40S (B), step 2: eluted pool: from RPC1 (C) and step 3: eluted pool from RPC2, final product (D).

Summary for insulin precursor purification

The added capture step using WorkBeads 40S reduced the loaded impurities on the first RPC column from 27.5% to 12%. This improvement should significantly reduce the fouling of the RPC column and the need for cleaning-in-place, prolonging the lifetime of the RPC resin. The final purity for the process was 99.7%, well above the target of 99%.

WorkBeads 40S gave higher purity than Capto SP ImpRes in the introduced capture step due to that WorkBeads 40S often gives different selectivities as compared to other cation exchange resins.

Summary

Since impurities from the peptide feed are prone to cause fouling of the commonly employed silica based high-performance RPC resins, the lifetimes of RPC columns are limited. The RPC silica resins are not stable enough to tolerate the required sodium hydroxide washes needed to remove the impurities bound to the resin. By introducing an upstream CIEX step before the RPC step(s), the burden of impurities is significantly reduced. This was shown for two different crude peptide feeds; a solid phase synthesized peptide and a recombinant human insulin precursor expressed in a bacterial host. These pose different challenges when it comes to impurity and contaminant loads on the resins. The recombinant insulin precursor feed imposes a heavy bioburden which causes extensive fouling of high-resolution resins.

Thus, the advantage of introducing CIEX as an orthogonal purification step is not only enhanced purity of the final peptide product, but also removal of significant impurities, *e.g.* bioburden from the feed. Since the agarose-based CIEX resin can tolerate a high sodium hydroxide concentration, extensive cleaning-in-place can be performed and the resin can be regenerated to its original state of performance.

WorkBeads 40S showed superior performance regarding capacity and selectivity (purity/yield) compared to Capto SP ImpRes, and additionally has excellent mass transfer properties and no hindered diffusion. This was shown in both cases presented in this application note. To conclude, WorkBeads 40S is an excellent choice to incorporate in peptide purification processes as a capture step prior to the RPC steps, and can be used in both lab-scale and industrial-scale purification.

The results demonstrate the significant enhancement of purity using an agarose-based orthogonal cation exchange step before the final polishing by RPC. Further investigations will promote understanding of the gain in productivity for the RPC step from being able to load a larger feed, reduce fouling and increase column lifetime.

Depending on peptide sequence and desired conditions, either an CIEX resin or an AIEX resin may be employed as a first capture step. WorkBeads 40Q is an AIEX resin with the same base matrix as WorkBeads 40S, but with positively charged quaternary amine ligands. Both WorkBeads 40Q and WorkBeads 40S resins are excellent for research- and industrial-scale purification of peptides.

Ordering information

Visit www. bio-works.com for information regarding all WorkBeads resins.

Orders: sales@bio-works.com or contact your local distributor.

For more information about local distributor and products visit www.bio-works.com or contact us at info@bio-works.com

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Bio-Works, Virdings allé 18, 754 50 Uppsala, Sweden. For local office contact information, visit bio-works.com/contact.

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