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Isolation and Characterization of Chromium- and Cobalt-Binding Peptides

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Submitted in fulfillment of the Thesis Requirement for Bachelor of the Arts in Honors in Chemistry-Biochemistry

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Abstract

Metal substitution has been proposed as an aid in the characterization of the structure of low-molecular-weight chromium-binding substance (LMWCr), which appears to be the biologically active chromium species. Preparations of bovine liver were diced and suspended in an isolation solution containing either a cobalt or a chromium salt. Peptide samples from these preparations were isolated by precipitation and centrifugation and purified by liquid chromatography. Several methods were used to analyze these samples, which may contain LMWCr or the cobalt-substituted form (LMWCo), in order to determine their purity, molecular weight (MW), and metal-to-peptide ratio. Methods used to determine MW included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and high performance liquid chromatography (HPLC). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) and fluorescence spectrophotometry were used to determine the metal-to-peptide ratio. The MW of the peptide appears to be between 3000 and 6000, and the metal-to-peptide ratio appears to be between 3.3 and 3.7 (previous reports suggest that LMWCr contains about 4 Cr(III) ions). Amino acid analyses have shown both peptides to contain significant amounts asp, glu, gly, and cys. However, the cobalt peptide has higher concentrations of cys than anticipated and the chromium peptide also has large amounts of ser, ala, lys, and pro. Initial attempts at N-terminal sequencing of the cobalt peptide have been unsuccessful.
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Introduction

Since the late 1950's, scientists have known that chromium is an essential trace element and that in its biologically active form it promotes the uptake of glucose in mammalian cells.\(^1\) A deficiency of this element has been known to cause glucose intolerance\(^1\) and chromium supplements have been shown to decrease plasma triglyceride and cholesterol levels in rats.\(^2\) However, no one has yet been able to determine the exact structure, function, and mode of action of any chromium-containing biomolecule, making chromium unique among the first-row transition elements.\(^3\)

Until the mid-1990's, it was assumed that a small molecule known as glucose tolerance factor (GTF) was the biologically active species containing chromium.\(^4\) Found originally in porcine kidney and later isolated almost exclusively from yeast, this material reversed the effects of chromium deficiency when fed to rats.\(^5\) Yeast GTF is known to contain the chromium(III) ion, glutamate, glycine, a sulfur-containing amino acid of uncertain identity,\(^5\) and perhaps nicotinic acid, although this has been questioned.\(^6\) Glutathione had also been proposed to be present, but this is impossible to determine, as the acid hydrolysis used to isolate GTF would have destroyed this part of the molecule.\(^7\) Unfortunately, recent evidence suggests that it only provides an easily absorbable form of chromium ion and is not in itself biologically active.\(^3,7\)

The so-called low-molecular-weight chromium-binding substance (LMWCr) is a much better candidate for the biologically-active chromium species. An oligopeptide reported to have a mass of about 1500 Daltons,\(^8,9\) LMWCr is believed to contain 2 aspartyl, 2 glycyl, 2 cystenyl, and 4 glutamyl residues, as well as 4 chromium(III) ions, and perhaps one or more organic cofactors, at least one of which may be aromatic.\(^5\) LMWCr has been found to stimulate the insulin receptor protein tyrosine kinase, increasing the kinase's activity approximately eightfold.\(^10,11\) Similarities between GTF and LMWCr have raised speculation that GTF may in fact be a product of the hydrolysis of crude LMWCr, although GTF is not produced by the hydrolysis of the pure substance.\(^7\)

Many attempts have been made to isolate and characterize LMWCr.\(^8,9,12-14\) Unfortunately, the chromium(III) ion is paramagnetic, which makes it difficult to use nuclear magnetic resonance (NMR) to collect and interpret data about the holo (metal-containing) form of LMWCr.\(^3\) At the same time, NMR studies on the apo (metal-free) form of the peptide have not been attempted, because the apo-peptide cannot be isolated in sufficient quantities (1-5 micromoles) for NMR experiments.\(^8\) In addition, for reasons related to chromium complex-formation, automated N-terminal sequencing has been unsuccessful in determining the amino acid sequence of LMWCr;\(^5\) and the apopeptide is not stable under the conditions required for sequencing.\(^8\)
The cobalt(III) ion is diamagnetic and has been suggested as a substitute for the chromium(III) centers in this oligopeptide, under the assumption that it should bind to LMWCr in much the same way as the chromium(III) ion, since both metal ions are first-row transition elements of approximately the same size and with the same valences.\textsuperscript{10} This seems a promising route toward structure characterization by NMR spectroscopy, especially since there have been reports of cobalt reconstitution of apo-LMWCr.\textsuperscript{10} As more than 90\% of bovine liver LMWCr is in the apo form,\textsuperscript{8} a simple addition of an easy-to-assimilate cobalt salt to a liver suspension should be able to accomplish such a reconstitution without the difficult task of removing the chromium ions that are already bound to the peptide.

Assuming that such a reconstituted peptide (which has been dubbed LMWCo) is stable, it will serve many useful purposes. First, as the cobalt(III) ion has no unpaired electrons, LMWCo would be diamagnetic and would allow for high resolution NMR structural studies of the substance. LMWCo may also prove somewhat easier to sequence than LMWCr if Co(III) centers are not substitutionally inert as Cr(III) centers are.\textsuperscript{8} If the sequence can be determined, then it will be fairly easy in the future to synthesize large quantities of this peptide for research, making time-consuming isolations unnecessary. The ultimate goal of this project is to use LMWCo to determine the primary structure of LMWCr so that it can be synthesized independently to enable further studies that can potentially determine other properties, such as LMWCr’s overall structure, binding sites, mode of action.
Materials

Bovine liver was purchased from Jason’s Butcher Shop (Albion, Maine). Denatured ethanol (CDA19), enzyme grade ammonium acetate, reagent grade potassium dichromate, enzyme grade sodium chloride, methanol, tricine, and dithiothreitol (DTT) were obtained from Fisher Biotech. The reagent grade nitric acid used for washing was purchased from Fisher Biotech or from ACROS. Reagent grade cobaltous chloride and sodium dodecyl sulfate (SDS) were purchased from J. T. Baker, Incorporated, as was trace metal grade nitric acid. All three chromatography resins (DEAE A-25, G-25, and G-15 Sephadex) were purchased from Pharmacia Biotech. Formamide, Stains-All (H213) and Brilliant Blue G (Coomassie Blue or Serva Blue) were obtained from Aldrich. Pepstatin A was purchased from Aldrich, Benzamide from Eastman Organic Chemicals, and aminoethylbenzenesulfonic acid (AEBSF) HCl from Sigma. CL-CAL2 ICP standardization solution was obtained from Spex Certi-Prep. Bacitracin, glacial acetic acid, and Tris base were obtained from ACROS, and the electrophoresis grade acrylamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), and sodium persulfate were obtained from Fisher Biotech. Glycerin was purchased from Fisher Chemicals. SYPRO Orange (#170-3120), Zinc Stain (#161-0440), and Silver Stain Plus (#161-0449), as well as gel filtration standards (#151-1901) for the size exclusion HPLC were purchased from BIO-RAD. Electrophoresis Polypeptide Standards (#161-0326) and Kaleidoscope Standards (#161-0325) were also purchased from BIO-RAD. Sodium phosphate was purchased from Fisher Chemicals.
Methods

This peptide was isolated and purified according to the method laid out by Davis and Vincent (Figure 1). Modifications were made when necessary, and these are indicated in the text. When not indicated otherwise, procedures are performed at ambient temperature.

Peptide Isolation from Bovine Liver. All precipitation and centrifugation procedures were performed at 4 °C, and all centrifugations were performed at 11325 x g in a J2HS centrifuge using a JA10 rotor unless otherwise indicated. The procedures below are for the first isolation of LMWCo, except where otherwise noted. The procedures for isolating LMWCr are exactly the same, except that the original cocktail contained 3.4 mM potassium dichromate instead of 6.8 mM cobalt(II) chloride. In neither case were oxidants or reductants used. In each of the isolations, the amount of time for centrifugation varied, so the minimum time is reported.

A pellet, believed to contain LMWCo, was obtained by following the procedures described in Davis and Vincent (which is a modification of those used by Yamato, Wada, and Ono). Approximately 1 kg of diced bovine liver was suspended in 1 L of an aqueous solution of 6.8 mM cobalt(II) chloride, containing a cocktail of protease inhibitors in the following concentrations: 3 mM benzamidine, 50 μM AEBSF HCl, and 350 nM pepstatin A. A Waring blender was used to homogenize the solution for 60 seconds at high speed. The homogenate was centrifuged for about an hour, the pellet (Pellet 1) was discarded, and a dropwise addition of ethanol was used to bring the supernatant (Supernatant 1) to 50% ethanol. During the ethanol precipitation, a magnetic stir-bar was constantly stirring the homogenate. After this was completed, the homogenate was stirred for another 12 hours, and then was centrifuged for at least one hour. The pellet (Pellet 2) was discarded, and using the same method as with the previous precipitation (although the supernatant, which had a very large volume, had to be divided into three parts), the supernatant (Supernatant 2) was brought to 90% ethanol. After the precipitation was complete, the flasks were stirred for one more day, and then the contents were divided into centrifuge bottles, and each portion was centrifuged for at least 30 min and the supernatant (Supernatant 3) discarded (specifically, some homogenate would be poured into each bottle, it would be centrifuged for ≥ 30 min, and then the supernatant was poured off and more homogenate would be poured in with the pellet).

Most of the pellet (Pellet 3) was scraped out and put in a 50 mL tube, and what could not be scraped out was rinsed out with water and put in another 50 mL tube. Both
Figure 1: Davis and Vincent
LMWCr Isolation Procedure

Homogenized Bovine Liver

Pellet 1 \(\rightarrow\) Supernatant 1

Pellet 2 \(\rightarrow\) Supernatant 2

Pellet 3 \(\rightarrow\) Supernatant 3

DEAE Sephadex A-25
(Anion Exchange Column)

Pooled Fractions

Sephadex G-25 (Size Exclusion Column)

Pooled Fractions

Sephadex G-15 (Size Exclusion Column)

Pooled Fractions
tubes were frozen in liquid nitrogen and lyophilized overnight (or longer), and the dry pellets were stored in a -20 °C freezer. After each centrifugation the volume of the supernatant and the mass of the wet pellet were measured and a sample of each was taken and stored at -20 °C for ICP analysis, except for the final centrifugation, where the dry pellet was weighed and a sample of it was stored.

**Chromatography.** All chromatography procedures were performed at 4 °C unless otherwise indicated. All pH values were measured by an Orion model 520A pH meter calibrated with pH 4.00, pH 7.00, and pH 10.00 buffers unless otherwise indicated. Buffers were brought to the proper pH by the addition of concentrated hydrochloric acid or 3 M sodium hydroxide. Solutions shall be referred to as Cr if they are involved in the isolation of the chromium bound peptide, or Co if they are involved in the cobalt bound form. Solutions which are to be eluted further through chromatography columns will be referred to by letters (e.g. Co A), while those which are to be subjected to characterization will be referred to by numbers (e.g. Co 1).

A DEAE Sephadex A-25 resin of about 200 mL, in a 0.2 M ammonium acetate buffer (pH 7.2) was shaken into a slurry and then poured in one motion into a 2.9 X 53 cm KONTES Flex-Column. The column was rinsed and packed with more 0.2 M buffer at a flow rate of 2.67 mL/min for 240 min on a BIO-RAD Econo Pump system (total volume = approx. 640 mL). A KONTES Flex-Column Flow Adapter was inserted and again the buffer was sent through the column at 2 mL/min for 120 min (total volume = 240 mL). In some cases, the flow adapter did not reach the top of the resin, leaving a gap of at least 2.6 cm, although in other cases the adapter was practically touching the top of the resin.

Pellet 3 was put in 0.2 M ammonium acetate buffer and partially dissolved (there was some solid left at the bottom). The dissolved portion was removed, and in some cases, an attempt at dissolving the remaining solid was made, but in no case was a solution from this attempt run through the column. The dissolved portion was filtered through a BIO-RAD 0.22 micron syringe filter and then loaded onto the column at 2 mL/min. Two hundred millimolar ammonium acetate buffer was allowed to flow through at 2 mL/min, with the eluent collected in a container for 4-9 hours, until it was clear that no more protein was eluting (an A₂₈₀ spectrophotometer measured the absorbance as the eluent flowed through to detect protein). During this entire time, a colored band (presumably containing the peptide of interest) was evident on the column, reddish-brown for LMWCo purifications, or black-green for LMWCr ones. Then, over the next 450-500 min (7.5-8.3 hours), a linear gradient from 0.2 M to 2 M ammonium acetate
buffer (with a constant pH of 7.2) was eluted into a fraction collector at 2 mL/min, where 9 mL fractions were collected every 4.5 min.

Fractions with large concentrations of both protein and metal were collected and combined into one or more pools. In general, each pool consisted of fractions surrounding a particular peak in absorbance or metal concentration, or fractions in a readily identifiable section of an absorbance or metal concentration graph; when there were several peaks, there were several pools. These pools were saved for future purification and analysis.

The column was cleaned with a continuous flow of 2 M ammonium acetate buffer at 2 mL/min for at least 25 min and then brought to its previous buffer concentration by running 0.2 M buffer through it at 2 mL/min for at least 2 hours.

All pools from the chromium isolation and the second portion of the cobalt isolation were stored, and characterization work on these pools was begun. The first portion of the LMWCo purification was run through the G-25 and the G-15 column.

An Amicon 8400 ultrafilter with a YC05 membrane was used to concentrate the pools and to desalt (remove the buffer from) them, bringing each pool to a volume between 10 and 35 mL (the volume varied greatly from pool to pool, although in general, the volumes were smaller with later pools, as it became easier to use the ultrafilter with practice). This process also brought the buffer to a concentration of about 50 mM and the pH to approximately 6.5.

A Sephadex G-25 column was packed using the same technique as the A-25 column described above, except that the buffer was 50 mM ammonium acetate at a pH of 6.5. Co A was added to this column at 1 mL/min and then 50 mM ammonium acetate buffer (pH 6.5) was run through the column at 1 mL/min into a fraction collector at 9 min/fraction (9 mL/fraction) for about 7 hours, until no more protein was detected at 280 nm in the eluent. The absorbance was mistakenly not recorded for the first 2-3 hours of the elution, and so ultraviolet-visible analyses for the wavelengths between 180 and 820 nm were performed on fractions to determine their protein concentrations. Using this information, the resulting fractions were collected and combined into two pools, which were concentrated by ultrafiltration. During this procedure, > 50% of one pool (Co C) was lost due to a spill.

Using the same technique and buffer as with the G-25 column, a Sephadex G-15 column was packed. Each of the two pools from the G-25 column was run through the column separately for about 5-6 hours at 1 mL/min until no more protein showed up in the eluent, or until all color was out of the column (after about 3 hours, the flow rate was switched to 0.75 mL/min for the first pool run through the G-15 column [Co C], and was left at that rate for the remaining time; the flow rate was a constant 1 mL/min for the
second pool). Due to errors in setting the absorbance detector, only the ICP results were used to determine which fractions contained cobalt. The fractions from the G-15 chromatography of the Co C pool were collected and combined to form the Co 1 pool, while the fractions from the G-15 chromatography of the Co B pool were collected and combined to form two pools: Co 2 and Co 3. These pools were concentrated by ultrafiltration.

**Microwave Digestion.** Samples from various pellets from the precipitation and centrifugation steps in the first isolation of LMWCo were digested by means of a CEM Microwave Sample Preparation System with the Lined Digestion Accessory Set. A known mass of each sample (less than 0.5 g) was weighed into each Teflon vessel, and 5 mL of 70% nitric acid (trace metal grade) and 2 mL of water were added. The Teflon vessels were set up in the lined digestion vessels, and a three-stage program was initiated in a MDS-2000 microwave. Digestion was also performed on the first supernatant. To prevent overheating, all six vessels were used even when there were fewer than six samples to digest. Any vessels that did not contain a sample contained water.

**Inductively-Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).** A Leeman Labs, Inc. PS1000 ICP was calibrated to detect cobalt at a wavelength of 228.62 nm or to detect chromium at 267.720 nm using CL-CAL2 standards in 5% trace metal grade nitric acid. In these analyses, standards of 0, 50, 100, 250, 1000 and sometimes 2000 or 2500 parts per billion (ppb) were used. The ICP instrument was set to measure at peak wavelengths for 5 seconds and at background for 5 seconds. Rinse and uptake time were both 60 seconds, and for each sample three measurements were averaged.

In all cases, appropriate dilutions were made of the analyzed samples. The cobalt or chromium concentration of each solution was determined by comparison with an appropriate standard curve.

**Electronic Spectra.** Electronic spectra analyses were collected on certain samples for characterization. Analyses was performed using a Hewlett Packard 8425A diode array spectrophotometer from 190-820 nm. Appropriate blanks were used for each sample.

**Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE).** A fluorescamine staining method was used to visualize the isolated peptides on a Tris-tricine SDS-PAGE gel (30:1 acrylamide: bisacrylamide) in order to determine their MW.
and purity. The gels were 1.5 mm thick and 100 mm wide. Each gel contained 2 layers: a 55 mm separating gel, composed of 16.5% (w/v) acrylamide/bisacrylamide, 1 M Tris, and 5.83% glycerol; and a 7 mm stacking gel, composed of 4.08% acrylamide/bisacrylamide and 7.44% Tris. After the gels had set, they were placed in a Mighty Small II gel apparatus and immersed in running buffer (1M Tris, 1M Tricine, 5% sodium dodecyl sulfate (SDS) buffer, (pH 8.75)).

When necessary, peptide samples were dried in a Savant Speed-Vac Concentrator to reduce volume. Fifty microliters of boric acid (pH 9.0) and 30 µL of an acetone solution containing 0.1 mg/mL fluorescamine were added to these peptide samples, as well as to BIO-RAD Polypeptide Standards (PPS) and BIO-RAD Kaleidoscope Polypeptide Standards (KPS). After reacting for two min, samples were dried under vacuum. To each sample was added 10-25 µL of SDS-PAGE loading buffer (1.33% (w/v) SDS, 13.3% (v/v) 1.0 M Tris HCl, 21.6% (v/v) glycerol, 2% (w/v) dithiothreitol). Preparations were heated at 40 ºC for either 30 min or 5 min to observe the effects of heating the peptide for different lengths of time.

The gels were run at 100 V until the Brilliant Blue G dye was near the bottom of the gel, at which point the electrophoresis was stopped and the protein was detected by fluorescence. This was done using a Fotodyne Foto/UV 26 light source, and the image was recorded with a Fotodyne Foto Analyst Investigator with an ethidium bromide filter.

**High Performance Liquid Chromatography (HPLC).** Peptide samples were eluted through a Shodex SB-802HQ or a Shodex SB-803HQ HPLC column and the absorbance converted into electric potential and measured by a Knauer Variable Wavelength Monitor. The wavelength monitor was set to measure absorbance with a time constant of 0.15 and a range of 1.28 at various wavelengths. Samples were filtered prior to injection using an Osmonics, Inc. 0.22 µm nylon microfuge centrifuge filter (Catalog #CFR0201550). BIO-RAD Gel Filtration Standards (#151-1901) were prepared according to supplier protocols. A 50 mM sodium chloride, 50 mM sodium phosphate buffer (pH 7.0) was ramped from flow rate of 0 µL per min to 500 µL per min and then run through the column at a constant rate of 500 µL per min. Twenty microliters of sample were injected onto the column. Immediately the software began recording the data from the wavelength monitor. The absorbance of the eluent at a single wavelength in the 190-600 nm range was measured over a period of 45 or 60 min. Whenever the buffer flow was ramped from 0 µL/min to 0.5 µL/min gel filtration standards and blanks were run.
**Dialysis.** All dialysis procedures were conducted at 4 °C unless otherwise indicated. Approximately 10 mL of a Cr or Co sample was loaded into a 3-4 cm length of Spectra/Por Cellulose Ester 500 molecular weight cut-off (MWCO) dialysis tubing with Spectra/Por closures. The tubing was immersed in 1.0 L of deionized water for at least four hours, with constant stirring. After four hours (or longer), the water was replaced, until the tubing had been immersed a total of four times. After the fourth time, the tubing contents were transferred to a falcon tube and stored at -20 °C.

**Ultrafiltration.** All ultrafiltration procedures were conducted at 4 °C unless otherwise indicated. Buffered Cr or Co samples were ultrafiltered at 30-55 psi in an Amicon 8400 Concentrator with a YC05 MWCO 500 filter. Samples were diluted with a solution of the desired buffer concentration and pH, or with deionized water, and then the solution was forced through the filter to decrease the volume while retaining the protein. Between 0.3 and 1 mL of liquid passed through the filter per min. The process was repeated as many times as necessary to get the desired buffer concentration, pH, and volume. The final samples were transferred to a tube and stored at -20 °C.

**Fluorescence Spectrophotometry.** The peptide concentrations of Co and Cr samples were determined through the use of fluorescence spectrophotometry. A 100 µL sample was mixed with 1000 µL of 0.2 M boric acid buffer (pH 9.0) and 500 µL of 0.1 mg/mL fluorescamine in acetone and was allowed to react for 1-2 min. Fluorescence was monitored in a quartz cuvette in a Perkin-Elmer 650-10S fluorescence spectrophotometer attached to a voltmeter. The spectrophotometer was set at an excitation wavelength of 385 nm and an emission wavelength of 495 nm. The excitation slit width was 2 nm and the emission slit width was 20 nm. Response, mode, and gain were set on "Norm," and the sensitivity and the zero were adjusted beforehand so that a water blank gave a signal near 0 V and the most concentrated standard gave a signal between 0.50 and 1.00 V. This method should give fluorescence which is proportional to the concentration of primary amines in the solution.

**Amino Acid Analysis.** One milliliter of ultrafiltration-desalted Co 1 and Cr 1 (containing approximately 13.5 and 800 mg of peptide, respectively) were sent to Commonwealth Biotechnologies, Inc. for analysis. Amino acid content was determined by routine hydrolysis and by hydrolysis with dithiopropionic acid exchange. An attempt was made to determine amino acid sequence by Edman (N-terminus) degradation.
Results and Discussion

Isolation and Purification of Cobalt-Bound Peptide

Isolations were performed according to the method of Davis and Vincent (Figure 1). An overview review of the chromatography purifications done for this report is included in Figure 2.

Isolation by Precipitation. The cobalt concentrations of all of the products from the cobalt isolation were determined in order to determine the potential for isolating a cobalt-bound peptide. Portions of each pellet and each supernatant from the cobalt isolation were digested and cobalt concentration was measured by ICP-AES. The final pellet from the precipitation and centrifugation steps for the cobalt isolation contained about 330 µmol (19 mg) of cobalt, or about 5% of the amount originally added to the bovine liver (Figure 3). The first precipitation step removed about half of the cobalt and the second removed about two-fifths of what remained, according to ICP data. About one-quarter of the cobalt could not be accounted for, which could be explained in several ways. The extremely dilute nature of the third supernatant made it difficult to get an accurate reading of its cobalt content, and it is likely that the first two pellets, which were not lyophilized, were not uniform in water content, so the samples used to determine cobalt content may not have been representative of each entire pellet.

The total mass of pellet 3 was approximately 5 g, and it retained about 5% of the cobalt initially added to the 1 kg of bovine liver, meaning that the concentration of cobalt in the pellet is approximately 10 times that of the liver as a whole. This implies that the Davis and Vincent procedure is successful at isolating some substance or substances from bovine liver that bind to cobalt.

A-25 Anion Exchange Column. As LMWCr is has been characterized as an unusually anionic peptide, anion exchange can be used to separate it from the majority of other peptides in the liver. The third pellet from the cobalt isolation was divided into two portions. Both portions of the pellet were dissolved and then eluted through a DEAE Sephadex A-25 anion exchange column.

The graph of cobalt concentration in the eluted fractions from the first portion of the pellet (Figure 4) has peaks at elution volumes of approximately 410 mL and 490 mL (the beginning of the concentration gradient was 0 mL). The peaks were reasonably well resolved, although there appears to be some overlap at around 450 mL. Unfortunately, there are no A_{280} data to which to compare the metal concentration data. The fractions
Figure 2: An Overview of the Various Chromatographic Purifications

LMWCo isolation
Homogenations/Centrifugations
Pellet 3
Co A
Co B
Co 2
Co 1
Co C
Co 3
Co 4
Co 5
A-25
G-25
G-15

LMWCr isolation
Homogenations/Centrifugations
Pellet 3
Cr 1
Cr 2
Cr 3
A-25
Figure 3: Location of Cobalt during Isolation

Figure 3: Cobalt distribution in the products from the centrifugation and precipitation steps of the isolation. Percentages are calculated compared to the 6.8 mmol of cobalt originally added to the bovine liver. Metal content was determined by ICP-AES.
Figure 4: Normalized cobalt concentration data from the A-25 chromatography of the first portion of cobalt isolation Pellet 3. Metal concentrations were determined by ICP-AES. Dashed vertical lines indicate the volumes between which fractions were pooled.
between elution volumes of 351 mL and 522 mL (Co A - indicated by dashed vertical lines on the graph) were pooled and concentrated by ultrafiltration. It would have been better to have separated the peaks, but this was the first column that was run for this experiment, and it was presumed that if there were two different materials, they would be separated by the next column.

The normalized graphs of cobalt concentration and $A_{280}$ data for the A-25-eluted fractions from the second portion of the pellet (Figure 5) show correlation between cobalt concentration and peptide concentration for the middle two peaks, at elutions approximately 450 mL and 515 mL after the start of the gradient. There is also an earlier $A_{280}$ peak at 405 mL for which there is no metal data, and another $A_{280}$ peak at 620 mL which does not contain significant concentrations of cobalt. The peaks were not baseline resolved. The fractions between elution volumes of 423 and 477 mL were pooled (Co 4), as were those between 477 and 549 mL (Co 5). Each pool was concentrated by ultrafiltration.

**G-25 Size Exclusion Column.** G-25 chromatography has lower and upper exclusion limits of 1 kDa and 5 kDa, respectively, and was used as a means to separate LMWCo from larger peptides. Co A was brought to 50 mM ammonium acetate (pH 6.5) by ultrafiltration, and eluted through a G-25 size exclusion chromatography column. The graph of the cobalt concentrations of the fractions eluted from the G-25 column (Figure 6) shows a sharp peak at an elution volume of about 225 mL (the zero point occurred when the entire Co A sample had been added to the column). There is another peak at approximately 160 mL, but there is very poor resolution between the two peaks. The fractions between elution volumes of 108 and 180 mL were pooled (Co B), as were those between 180 mL and 270 mL (Co C). Both pools were concentrated by ultrafiltration.

**G-15 Size Exclusion Column.** G-15 chromatography has lower and upper exclusion limits of 100 Da and 1500 Da, respectively, and was used as a means to separate LMWCo from smaller peptides. First Co C and then Co B were eluted through a G-15 size exclusion chromatography column. The cobalt concentration graph of the fractions eluted from the G-15 chromatography of Co C (Figure 7) shows a single peak at around 125 mL elution volume. Although the peak is not narrow, there are no other peaks in the graph. Fractions between elution volumes of 90 and 153 mL were pooled (Co 1) and concentrated by ultrafiltration.
Figure 5: Normalized cobalt concentration data and normalized $A_{280}$ data from the A-25 chromatography of the second portion of cobalt isolation Pellet 3. Metal concentrations were determined by ICP-AES. Adjacent dashed vertical lines indicate the volumes between which fractions were pooled.
Figure 6: Cobalt Concentrations in the Eluent from the G-25 Chromatography of Co A

Figure 6: The eluent (Co A) from the A-25 column was brought to 50 mM NH$_4$OAc buffer (pH 6.5) and eluted through a G-25 size exclusion column. Metal concentration was determined by ICP-AES. Adjacent dashed vertical lines indicate the volumes between which fractions were pooled.
Figure 7: Cobalt Concentrations in the Eluent from the G-15 Chromatography of Co C

Figure 7: A pool of the eluent (Co C) from the G-25 column was eluted through a G-15 size exclusion column. Metal concentration was determined by ICP-AES. Dashed vertical lines indicate the volumes between which fractions were pooled.
The graph of cobalt concentrations of the fractions eluted from the G-15 chromatography of Co B (Figure 8) shows a single peak at an elution volume of about 90 mL. There appears to be some sort of shoulder starting at 108 mL, suggesting that there may be a second peak that is not well resolved. Fractions from elution volumes between 54 and 108 mL were pooled (Co 2) as were those between 108 and 162 mL (Co 3). Both pools were concentrated by ultrafiltration.

*Size Exclusion HPLC Analysis.* Size exclusion HPLC was used to determine the purity of the substance isolated in each sample. 20 μL portion of 50 mM ammonium acetate buffer was injected onto Shodex SB-802HQ and SB-803HQ columns. This ammonium acetate buffer in which all samples had been dissolved during chromatographic purification only produced a signal at one of the wavelengths (224 nm) which was studied (Figure 9). For the 802HQ column, the peaks were at elution times of 11.1, 16.5, and 23.7 min. For the 803HQ column, they were at 20.0, 21.2, 25.8, and 29.0 min. The Co 1 and Co 4 samples were each run through a Sephadex SB-802HQ column and the absorbance at 224 nm measured (Figure 10).

The peaks for Co 1 were at 11.1, 12.8, 16.6, and 23.8 min. All except for the peak at 12.8 can be accounted for by the buffer signal. The peaks for Co 4 were at 12.7, 16.6, and 23.8 min, with buffer signal accounting for all but the 12.7 min peak. There are two reasons for the absence of a peak at 11.1 min in Co 4 is twofold. First, the peak at 12.7 min was much broader than the corresponding peak at 12.8 min in Co 1, and HPLC was not able to resolve the two. Second, Co 4 may have had a higher ammonium acetate concentration, which tends to depress the 11.1 min signal (data not shown).

In both cases, there appears to be a single non-buffer peak at 12.7-12.8 min, suggesting that both samples are of similar composition and purity, with no significant difference in purity between the two samples. This suggests that, at least in the case of the cobalt isolation, the G-25 and G-15 chromatography columns are not needed for further purification, at least not if separate pools are obtained from the two peaks in the A-25 eluent. This is useful to know, as running extra columns diminishes the peptide yield. It may be possible that the use of the protease inhibitor cocktail, a recent addition to the isolation/purification procedure, has reduced the number of steps needed for purification, as the cocktail reduces the amount of extraneous polypeptides to be separated from LMWCr.

*Dialysis vs. Ultrafiltration.* Dialysis or ultrafiltration was used to remove the buffer from samples prior to some analyses. The results for Co 5 are typical for samples where portions have been desalted in both ways. Portions of Co 5 were dialyzed or
Figure 8: A pool of the eluent (Co B) from the G-25 column was eluted through a G-15 size exclusion column. Metal concentration was determined by ICP-AES. Adjacent dashed vertical lines indicate the volumes between which fractions were pooled.
Figure 9: HPLC results from 50 mM ammonium acetate blanks run through (A) SB-802HQ and (B) SB-803HQ Shodex HPLC columns. Twenty microliters of 50 mM ammonium acetate buffer were injected into each column and eluted through the column at a rate of 0.5 mL/min. The absorbance of the eluent was measured at 224 nm.
Figure 10: HPLC results of (A) Co 1 and (B) Co 4 injections on a Shodex SB-802HQ HPLC column. Twenty microliters of each sample were eluted through the column at a rate of 0.5 mL/min. The absorbance of the eluent was monitored at 224 nm.
ultrafiltered. The resulting samples were eluted through a Shodex SB-803HQ HPLC column, and their absorbance at 224 nm monitored (Figure 11).

The signal for the buffered sample had peaks at elution times of 19.2, 21.3, and 25.8 min. The 21.3 and 25.8 min signals are accounted for by the buffer components, while the 19.2 min signal represents the isolated substance. The dialyzed sample has peaks at 19.2, 21.2, 25.1, and 30.2 min, with the largest buffer peak (21.2 min) approximately one-third as large (normalized for the 19.2 min peak) as it is in the buffered sample. The 25.1 and 30.2 min peaks are relatively small and appear to represent buffer peaks. The ultrafiltered sample has only one measurable peak at 19.2 min.

The results indicate that ultrafiltration is superior to dialysis as a means of desalting cobalt samples, as ultrafiltration consistently reduces the buffer concentration to levels that are not detectable at 224 nm.

Isolation and Purification of Chromium-Bound Peptide

Isolation. The third pellet from the chromium isolation was eluted through a DEAE Sephadex A-25 anion exchange column. The normalized graphs of chromium concentration and A_{280} data for the eluted fractions (Figure 12) show chromium peaks at elution volumes of 490 mL, 540 mL, and 575 mL, (approximately 54%, 60%, and 64% of the way through the gradient). The graphs also show peptide peaks at 490 mL, 530 mL, 560 mL, 700 mL, and 860 mL (54%, 59%, 62%, 78%, and 95% of the way through the gradient). These results have some similarity to the results of Yamato et al., where peptide peaks appeared 48%, 57%, and 64% of the way through the gradient, and where there was a single chromium peak extending from 55% to 70% of the way through the gradient. The larger number of chromium peaks in this study may be due to differences in preparation (Yamamoto et al. homogenated rabbit liver and did not use protease inhibitors), or may indicate better resolution of chromium peaks.

There is some correlation between the first three peptide peaks and the three chromium peaks, with the best correlation occurring in the first peak. Part of the reason for the poor correlation of the second two peaks may be due to the fact that the A_{280} data is continuous, while chromium concentration data is taken at discrete 18 mL intervals. The first chromium peak was well resolved, and the second two peaks were reasonably well resolved, although there was definitely some overlap in the troughs at 520 and 560 mL. The fractions between elution volumes of 441 and 540 mL (49% to 60%) were pooled (Cr 1), as were those between elution volumes of 540 and 603 mL (60% to 67%);
Figure 11: HPLC results of eluting Co 5 injections on a Shodex SB-803HQ HPLC column. Twenty microliter aliquots of Co 5, one buffered (A), one dialyzed (B), and one ultrafiltered (C) were eluted from an HPLC column at a rate of 0.5 mL/min. The absorbance of the eluent was monitored at 224 nm.
Figure 12: Normalized chromium concentration data and normalized $A_{280}$ data from the A-25 chromatography of the chromium isolation Pellet 3. Metal concentrations were determined by ICP-AES. Adjacent dashed vertical lines indicate the volumes between which fractions were pooled.
Cr 2), and 603 and 675 mL (67% to 75%; Cr 3). The fractions pooled by Yamamato et al. were from 55% to 70%. Each pool was concentrated by ultrafiltration.

It should be noted that Cr 1 contains half of the second chromium peak and all of the second peptide peak. Pooling the fractions between 441 mL and 513 mL would probably have been better, as this would have isolated only one peak. From this data it seems likely that there will be impurities in the Cr 1 sample. Similarly, the Cr 2 sample, which includes all of the third chromium peak and half of the second, likely contains more than one substance as well.

**Size Exclusion HPLC Analysis.** Cr 1 was injected onto a Shodex SB-803HQ column and the absorbance at 224 nm measured (Figure 13). For the Cr 1 sample analyzed on the Shodex SB 803HQ column peaks occurred at elution times of 18.6, 21.5 and 25.8 min with A224 monitoring. All except for the peak at 18.6 can be accounted for by the buffer signal. The peaks were well resolved, although the peak at 18.6 is rather broad and has a shoulder at about 15.2 min. This shoulder could be due to the problems with the pooling of the A-25 fractions that was mentioned earlier.

These results are further confirmed by examining the signal from desalted (by ultrafiltration) Cr 1 (Figure 13B). There is only one peak, at 18.7 min, with the same shoulder at about 15.0 min. The peaks at 21.5 and 25.8 min are gone, confirming that they were caused by the buffer.

Other than the shoulder and the buffer, Cr 1 seems to contain a relatively pure substance. The broad peak suggests either that the samples are too concentrated and further HPLC work needs to be done on diluted samples. This may also produce better resolution between the shoulder and the peak at 18.6 min. The only concern for the purity of Cr 1 other than the shoulder is the possibility that the rather broad peak contains more than one substance. If these features do indicate impurity, it is unclear how much the purity would have been improved had the chromium A-25 fraction pooling not included parts of two chromium peaks (Figure 12).

**Characterization of Isolated Peptides.**

**Electronic Spectra.** Electronic spectra of each sample were used to determine the \( \lambda_{\text{max}} \) values for each sample, and to compare the Cr sample spectra to the spectrum for LMWCr as recorded in the literature. A comparison of the absorbance signals of Co 4 and Co 5 at wavelengths between 200 and 600 nm indicates that Co 4 and Co 5 have similar electronic transitions (Figure 14). Both samples have \( \lambda_{\text{max}} \) values around 265 nm, 350 nm, and 440 nm. It should also be noted that the Co 4 peak at 439 nm was rather
Figure 13: HPLC results of Cr 1 and Cr 2 injections on a Shodex SB-803HQ HPLC column. Twenty microliter aliquots of (A) buffered Cr 1, (B) ultrafiltered Cr 1, and (C) ultrafiltered Cr 2 were eluted from an HPLC column at a rate of 0.5 mL/min. The absorbance of each eluent was monitored at 224 nm.
Figure 14: The electronic spectra of Co 4 and Co 5, measured against appropriate blanks, were plotted. The two spectra were normalized at the point of greatest absorbance. Wavelengths of absorbance maxima are labeled on the graph.
weak, resembling a shoulder. Overall the results suggest that Co 4 and Co 5 contain a similar substance, although Co 4 may be slightly less pure than Co 5. Comparison to literature values is not possible, as no such values exist.

The electronic spectra of Cr 1 and Cr 2 from 300 to 800 nm were compared to literature results for LMWCr\(^\text{8}\) (Figure 15). The literature sample has peaks at 411 and 577 nm (although the literature\(^\text{8}\) lists the first peak as being at 394 nm, the data that was displayed belies that). Cr 1 appears to have almost exactly the same spectrum, with peaks at 410 and 578 nm. Cr 2 has a similar spectrum, with a shoulder at about 400 nm and a peak at 573 nm. Both Cr samples appear to lack shoulders which Davis and Vincent\(^\text{8}\) had reported at 316 and 336 nm. The similarity between Cr 2 and the literature suggests that Cr 2 may contain some LMWCr, but may also contain significant impurities. Cr 1 however appears to contain LMWCr, and to have no major impurities that absorb between 300 and 800 nm.

**Size Exclusion HPLC Analysis.** HPLC was used at various wavelengths to determine whether the \(\lambda_{\text{max}}\) values from the electronic spectrum of each sample were caused by a single species. Co 4 was run through a Sephadex SB-802HQ three times, with its signal detected at 224 nm, 280 nm, and 350 nm, respectively (Figure 16). The signal at 224 nm, indicating the peptide backbone, had a non-buffer peak at an elution time of approximately 12.7 min, as discussed above. The signal at 280 nm, presumably indicating the organic cofactor predicted by Davis and Vincent,\(^\text{8}\) had a peak at 12.7 min and a small, poorly resolved peak at 14.2 min, that had also appeared as a shoulder at 224 nm. The signal at 350 nm, which probably represents a cobalt-centered absorbance, was also at 12.6 min with a small peak at 14.1 min. These results indicate that the major component of the sample is a single substance which absorbs at 224 nm, 280 nm, and 350 nm, and therefore that this substance may contain peptide, an aromatic cofactor, and cobalt. Furthermore, it appears that there exists another, similar substance in the sample, which has a lower molecular weight and therefore elutes through the column more slowly. This may be a degradation product of the major component. There appear to be no other substances in the sample.

Cr 1 was run through a Sephadex SB-802HQ column three times, with its signal detected at 224 nm, 410 nm, and 578 nm, respectively (Figure 17). The signal at 224 nm, indicating the peptide backbone, had peaks at elution times of 11.5, 12.2, 16.8, and 23.8 min, where all but the 12.2 min peak could be accounted for by the buffer signal. The signals at 410 and 578 nm, presumably indicating two different chromium-centered absorbances, both produced signals with a single peak, at 11.4 min for 410 nm and at 12.1 min for 578 nm. However, at 410 nm it looks as if there are two peaks very close
Figure 15: The electronic spectra of Cr 1 and Cr 2, against appropriate blanks, were plotted. The two spectra were normalized at the point of greatest absorbance and plotted along with data from the literature. Wavelengths of absorbance maxima are labeled on the graph.
Figure 16: HPLC results from Co 4 injections on a Shodex SB-802HQ HPLC column. Twenty microliter aliquots of Co 4 were eluted at a rate of 0.5 mL/min. The absorbance of the eluent was monitored at (A) 224 nm, (B) 280 nm, and (C) 350 nm.
Figure 17: HPLC results of Cr₁ injections on a Shodex SB-802HQ HPLC column. Twenty microliter aliquots of buffered Cr₁ were eluted at a rate of 0.5 mL/min. The absorbance was measured at (A) 224 nm, (B) 410 nm, and (C) 578 nm.

11.5  12.2  (peptide)  16.8  23.8

Figure 17A

11.5

Figure 17B

12.1

Figure 17C

(min)
together, and the peaks from all three graphs seem to cover the same general space (11 to 14 min), so it is likely that the same substance is causing at least part of the peak for all 3 wavelengths. It is also possible that there is another substance that absorbs at 224 nm and 410 nm that is contained in Cr 1 and which elutes at about 11.4-11.5 min. This might in fact be the shoulder that appears on the 803HQ graphs.

**Metal-to-Peptide Ratio.** In order to characterize the peptide samples, ICP-AES and fluorescence spectrophotometry were used to determine the ratio of metal to peptide. Metal and peptide concentrations and the resulting ratios are displayed in Table 1.

Spectrophotometry using fluorescamine, a reagent that fluoresces only when reacted with primary amines,\(^6\) was performed on the peptide samples. The concentration of free amine was determined by comparison with a glycine standard curve (Figure 18). LMWCr, as identified by Davis and Vincent,\(^8\) does not have any amino acids that contain amino side chains, although this may be in doubt.\(^7\) Therefore, free amine concentration should be equivalent to peptide concentration. Signals of the standards were measured both before and after the peptide samples, to test for decay of signal. For the Co 4 and Co 5 measurements, there was decay, but curves taken before and after the measurement of the peptide signal were both very linear and so peptide concentration was calculated as the average of the results from the two curves.

Table 1 displays the metal concentration, the peptide (free amine) concentration and the molar metal-to-peptide ratio for the Co 4, Co 5, Cr 1, and Cr 2 samples, along with standard deviation determinations. Co 4 has a metal-to-peptide ratio near 3.5, the literature value reported for LMWCr.\(^8\) Co 5 had a ratio of 5.8, indicating that Co 5 either does not contain the desired peptide, that it contains impurities, or that it had lost peptide due to degradation. For both samples the standard deviation was less than or equal to 0.2, indicating a high degree of precision in these measurements.

The standard curve for Cr 1 did not decay, but that used for Cr 2 did, and so for Cr 2 the free amine concentration was determined by averaging the results from standard curves taken before and after the detection of the peptide signal. Cr 1 and Cr 2 both have metal-to-peptide ratios at or near 3.5, which is the literature value.\(^8\) In each case the standard deviation was less than or equal to 0.2, indicating a high degree of precision in these measurements.

**Molecular Weight**

Molecular weight was measured in two ways: through SDS-PAGE and through
HPLC. In both cases, the molecular weight was determined by comparing the peptide to a set of standards. Molecular weight data is compiled in Table 2.

**SDS-PAGE.** With SDS-PAGE, the logarithm of molecular weight should vary linearly with the distance traveled, with lower MW substances traveling greater distances. A standard curve was made from BIO-RAD polypeptide standards (#161-0326, Figure 19), except for bacitracin, which did not fit well onto the curve. Co 4 and Co 5 samples were run on two gels; in the first they had been heated at 40°C for 30 min prior to running, in the second they had been heated only 5 min. The brightest Co bands were A and B (Figure 19). From the standard curve the molecular mass of each sample was estimated to be 4500 Da.

The Co samples that were heated for 30 min (data not shown) also had light bands corresponding to MWs of 650 to 750 and 2300 to 2500. As these bands are not present in the gel which was heated for 5 min, they probably represent products of a breakdown in the structure of the peptides caused by heating them in SDS sample buffer.

According to Davis and Vincent, the peptide should appear at about the same position as bacitracin. This did not appear to be the case here, suggesting that the peptides which have been isolated are not the cobalt-substituted version of the Cr-peptide isolated by Davis and Vincent.

Cr 1 and Cr 2 were also run on two gels, having been heated in the same manner as the Co samples. From the standard data, these Cr samples contain a major component (C, D in Figure 19) with an estimated MW of 4500.

It should be noted that in both gels Cr 1 and Cr 2 appears to have a faint band at E and F, which correspond respectively to MWs of 15000 and 16000. This may represent the material in the shoulder/peak present in the HPLC chromatograms (Figures 13B, 13C). The Cr samples which were heated for 30 min (data not shown) also have bands corresponding to MWs of 650 to 750 and 2300 to 2500, similar to those from the Co samples. Like the Co bands, they probably represent products of a breakdown in the structure of the peptides caused by heating them in SDS sample buffer.

This peptide did not appear at the same position as bacitracin, but rather the same position as bands A and B, suggesting that the peptides which have been isolated are not the same as those isolated by Davis and Vincent.

**HPLC.** With size exclusion HPLC, the logarithm of molecular weight varies with the time of elution, with higher MW substances eluting faster. A standard curve was made using BIO-RAD gel filtration standards (#151-1901, Figure 20). All but the lightest standard (vitamin B$_12$, MW =1350) appeared to be collinear. The major
Table 1: Fluorescamine fluorescence spectrophotometry and ICP-AES were used to determine the primary amine and metal concentrations, respectively, of the Co 4, Co 5, Cr 1, and Cr 2 peptide samples. This information was used to determine the molar metal:peptide ratio of each sample, as well as a standard deviation.

<table>
<thead>
<tr>
<th>Ultrafiltered sample</th>
<th>[Peptide] (µM)</th>
<th>[Metal] in sample (µM)</th>
<th>Metal:Peptide Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co 4</td>
<td>9 +/- 1</td>
<td>30 +/- 2</td>
<td>3.3 +/- 0.2</td>
</tr>
<tr>
<td>Co 5</td>
<td>6.0 +/- 0.5</td>
<td>34 +/- 0</td>
<td>5.8 +/- 0.1</td>
</tr>
<tr>
<td>Cr 1</td>
<td>549 +/- 27</td>
<td>2008 +/- 49</td>
<td>3.7 +/- 0.1</td>
</tr>
<tr>
<td>Cr 2</td>
<td>120 +/- 1</td>
<td>424 +/- 14</td>
<td>3.53 +/- 0.04</td>
</tr>
</tbody>
</table>

Table 2: Molecular weights of the samples Co 4, Co 5, Cr 1, and Cr 2 were measured by HPLC and SDS-PAGE, in each case by comparison with a standard curve. For the samples where there is more than one trial, an average was taken and a standard deviation is included. There is also a comparison of the MWs determined by the two methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPLC- determined MW</th>
<th>SDS-PAGE- determined MW</th>
<th>HPLC MW/SD (±/-)</th>
<th>SDS-PAGE MW</th>
<th>%SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co 4</td>
<td>3910</td>
<td>4500</td>
<td>0.51</td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>Co 5</td>
<td>3519</td>
<td>4500</td>
<td>0.23</td>
<td></td>
<td>0.78</td>
</tr>
<tr>
<td>Cr 1</td>
<td>5700</td>
<td>4500</td>
<td>3.51</td>
<td></td>
<td>1.27</td>
</tr>
<tr>
<td>Cr 2</td>
<td>3900</td>
<td>4500</td>
<td>-</td>
<td></td>
<td>0.87</td>
</tr>
</tbody>
</table>
Figure 18: Glycine concentration versus the signal obtained from measuring fluorescent emissions at 495 nm. Glycine standard curves such as this one were used to when determining free amine concentrations by the fluorescamine method. This curve uses 0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 50 μM, 75 μM, and 100 μM glycine standards.
Figure 19: SDS-PAGE (left) of fluorescamine-labeled (A) Co 4 , (B) Co 5, (C) Cr 1, and (D) Cr 2. One to five microgram samples of each pre-labeled peptide were loaded into wells on a 16.7% polyacrylamide Tris-tricine SDS-PAGE gel, along with Brilliant blue dye, Kaleidoscope standards, and polypeptide standards (G-L). Standard curve (right) created from the polypeptide standards and used to estimate the MW of the peptide bands and of the high MW bands found in the Cr samples (E and F). Bacitracin was not collinear with the other standards and was not included in the curve. The identities and MW of the standards are as follows: (G) Triosephosphate isomerase, 26625, (H) myoglobin, 16950, (I) α-Lactalbumin, 14437, (J) Aprotinin, 6512, (K) insulin, 3496, (L) bacitracin, 1423.
Figure 20: HPLC results of BIO-RAD Gel Filtration standards on a Shodex SD-803HQ HPLC column. Twenty microliter aliquots of standards were eluted at a rate of 0.5 mL/min. The absorbance was monitored at 224 nm. A standard curve of log MW vs. elution time was made from all of the standards except for Vitamin B$_{12}$, which was not collinear with the other points. The identities and MW of the standards are as follows: (A) thyroglobulin, 670000, (B) bovine gamma globulin, 158000, (C) chicken ovalbumin, 44000, (D) equine myoglobin, 17000, (E) vitamin B$_{12}$, 1350.
disadvantage of the exclusion of vitamin B₁₂ from the standard curve is that if the peptide being studied has an MW less than 17000, its MW must be extrapolated rather than interpolated.

This standard curve was used to make a determination of the MW of the Co 4 and Co 5 samples, using the values obtained from elutions on the Shodex SB-803HQ column (Figure 11C, Co 4 data not shown). In both cases, the standard deviation for the MW was less than 0.6%. The MWs of the samples are around 3500-4000, or within 25% of the MW determined by SDS-PAGE.

The standard solution used to make a determination of the MW of the Cr 1 and Cr 2 samples was the same as the one used for the Co samples; again the values were obtained from elutions on the Shodex SB-803HQ column (Figures 13B and 13C). In both cases the standard deviation for the MW was less than 4%. The MWs of the samples are around 3500-4000, or within 30% of the MW determined by SDS-PAGE.

The Cr 1 shoulder at about 15.5 min (Figure 13B) and the Cr 2 impurity at about 16.0 min (Figure 13C) would have MWs of about 75000 and 50000, respectively, using this standard curve. It should also be noted that an impurity of MW 15000 to 16000 would be expected elute at about 17.3 to 17.6 min on the Shodex SB-803HQ column, meaning that it could well be hidden by the broad peptide band.

Amino Acid Content. Table 3 contains data on amino acid content, defined here as the molar ratio of amino acid residue to free amine. Data from routine hydrolysis indicates that the major amino acids in Co 4 are glu, asp, gly, and cys, the same amino acids presumed to be in LMWCr. The concentrations of all other amino acids are quite low; the most abundant amino acid other than the predicted four is pro, which has a little more than one quarter the concentration of the least abundant of the four predicted acids (asp). The data from hydrolysis with dithiopropionic acid exchange (DAE) shows similar results.

However, the amino acid concentrations of these four amino acids do not conform to those found in LMWCr by Davis and Vincent. Both routine hydrolysis and hydrolysis with DAE indicate a asp:gly:glu ratio of 1:2:1.5, and both methods show very high concentrations of cys, 4 per peptide for routine hydrolysis, 7 for hydrolysis with DAE.

Interpreting these results is difficult. The cys concentration is uncertain, but it is likely to be much higher than that predicted. This may indicate a large amount of cys contamination, or the isolated peptide may consist mainly of cys residues. The gly ratios appear to indicate that there may be two primary amines per peptide, as that would make the gly per peptide ratio integral. As the method of detection of amino acids used here
Table 3: Amino acid analysis of Cr 1 and Co 4 samples. Acid hydrolysis, both routine and with dithiopropionic acid exchange (DAE), was used to determine the concentration of each amino acid in a sample. Peptide content was determined by dividing amino acid concentration by fluorescamine-determined peptide concentration. The four amino acids reported to be in LMWCr (glu, gly, asp, and cys) were specifically listed, as were any acids whose abundance was at least 0.80 in at least one sample by at least one method. The abundances of other amino acids were summed and listed as “other.”

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Content (^a)</th>
<th>Content (^b)</th>
<th>Co 4</th>
<th>Co 4</th>
<th>Cr 1</th>
<th>Cr 1</th>
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<tr>
<td>Asp</td>
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<td>2.15</td>
<td>1.16</td>
<td>1.14</td>
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<tr>
<td>Glu</td>
<td>4</td>
<td>4.47</td>
<td>1.93</td>
<td>1.93</td>
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<td>0.23</td>
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<td>0.09</td>
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<tr>
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<td>0.33</td>
<td>0.54</td>
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<td></td>
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<tr>
<td>Other</td>
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<td>1.54</td>
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<td>MW (estim.)</td>
<td>1084</td>
<td>1231</td>
<td>1004</td>
<td>1342</td>
<td>1056</td>
<td>1091</td>
</tr>
</tbody>
</table>
cannot distinguish asp from asn or glu from gln, it is possible that one of the supposedly acidic residues is actually the corresponding amine.

The analysis also included an estimate of the mass of the sample which was used to calculate the MW per primary amine. According to this data, the molecular mass for Co 4 was 1000-1350 Da per primary amine. While this is relatively close to the literature value, it is a fraction of that obtained by either HPLC or SDS-PAGE. This suggests either that the peptide contains more than one primary amine, or that two or more peptides form multi-subunit peptides that are not broken apart easily on a gel or HPLC column. It should be noted that on the SDS-PAGE where samples were heated for 30 minutes (data not shown), there were bands directly below C and D with estimated MWs of about 1500. This band may have been what was detected by Davis and Vincent in the Cr peptide.

The data from the routine hydrolysis appears to indicate that the major amino acids in Cr 1 are the same ones as are presumed to be in LMWCr, that is, glu, asp, gly, and cys. Of these, the least abundant is cys (0.66 per primary amine), which is only about 20% more abundant than the next most abundant amino acid, lys (0.55 per primary amine). The data from the hydrolysis with dithiopropionic acid exchange gives similar results, with the asp content only marginally higher than that of ala. Asp and glu seem to be approximately equally prevalent in Cr 1, and cys and gly are one third to one fourth as abundant. This may be due to asn and gln contamination, or to general contamination of the peptide, although such a contaminant would either need to be very close in MW to the isolated peptide in Cr 1 in order not to be resolved on SDS-PAGE, or to be non-reactive with fluorescamine. Cys concentrations are also lower than in Co 4. Assuming Cr 1 and Co 4 are the same peptide with different metals, this would indicate that the high cys levels in Co 4 are probably due to contamination.

The analysis also included an estimate of the mass of the sample which was used to calculate the MW per primary amine. According to this data, the MW for Co 4 was 1000-1350 Da per primary amine. While this is relatively close to the literature value, it is a fraction of that obtained by either HPLC or SDS-PAGE. This suggests either that the peptide contains more than one primary amine, or that two or more peptides form complexes that are not broken apart easily on a gel or HPLC column. It should be noted that on the SDS-PAGE where samples were heated for 30 minutes (data not shown), there were bands directly below C and D with estimated MWs of about 1500. This band may have been what was detected by Davis and Vincent in the Cr peptide.

For Cr 1, the data indicate a MW between 1000 and 1100. This is consistent with the Co 4 result for routine hydrolysis. This strengthens the hypothesis that both substances contain the same apopeptide. It also is consistent with the hypothesis that the
SDS-PAGE and HPLC MWs were due to two or more peptides forming complexes. The latter hypothesis is also supported by the fact that the Cr samples on the SDS-PAGE also displayed a second band of about 1500 Da when heated for 30 minutes.

**Amino Acid Sequence.** The attempt by Commonwealth Biotechnologies, Inc. to sequence this peptide through N-terminus Edman degradation was not successful. The first round of degradation produced high levels of gly, glu, and asp, but subsequent rounds did not produce significant quantities of any amino acid (data not shown).

**Mass Spectrometry.** Mass spectrometry has been suggested as a method for elucidating the structure of LMWCr. Peptide samples, some of which were dried by rotary evaporation, were sent to Los Alamos Labs in Los Alamos, New Mexico to be analyzed by matrix assisted laser desorption/ionization (MALDI) MS and by electrospray ionization (ESI) MS. The results (data not shown) were inconclusive.

**Comparison of Co- and Cr-Peptides**

**Metal Retention.** Metal retention can be useful as a proxy for peptide yield, and, if the Co- and Cr-peptides are the same, can indicate the binding affinity for each of the metals. The fractions that were collected from the A-25 chromatography of both of the portions of the cobalt pellet, representing the entire yield from 1 kg of bovine liver, retained a total of about 14 μmol (0.80 mg) of cobalt, compared to 157 mol (8.14 mg) of chromium from the 1 kg chromium isolation, also representing the entire yield from 1 kg of bovine liver (Table 4). The collected chromium fractions therefore have approximately 11 times as much metal as the cobalt ones, which would be consistent with the assumption that we are isolating a peptide which binds preferentially to chromium. If only Cr 1 is considered, then 96 μmol (5.0 mg) of chromium was retained. This is consistent with the results of the Davis and Vincent LMWCr isolation, where 551.2 μmol (28.66 mg) of chromium was retained after one A-25 chromatography of extract from 6 kg of liver, or about 91.87 μmol (4.777 mg) per kg.

**Purity of Samples.** Both the Co 4 and Cr 1 samples appear to contain one major species, and lesser amounts of at least one impurity. In each case, the large peak, which presumably contains the isolated substance, has a shoulder or minor peak very close to it. In the case of Co 4, the second peak is relatively small compared to the major peak (Figure 15), but in the case of Cr 1 the shoulder is large enough to worry about. It appears that no additional columns are needed for purification of the Co 4, but this is
Table 4: The metal content of the entire A-25 eluent from a 1 kg preparation of bovine liver with cobalt was determined, as was the metal content of the entire A-25 eluent from a 1 kg preparation of chromium, and from a specific pool from that eluent, Cr 1. A literature value for metal isolated per kg of liver for a chromium prep is also included in the table. Content is measured both in μmol and mg.

<table>
<thead>
<tr>
<th></th>
<th>[Metal] (μmol)</th>
<th>[Metal] (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt prep</td>
<td>14</td>
<td>0.8</td>
</tr>
<tr>
<td>Chromium Prep</td>
<td>157</td>
<td>8.14</td>
</tr>
<tr>
<td>Cr 1</td>
<td>96</td>
<td>5.0</td>
</tr>
<tr>
<td>Literature</td>
<td>91.87</td>
<td>4.777</td>
</tr>
</tbody>
</table>
uncertain for Cr I, as it depends on how much purity would have been improved if more
care had been taken when combining A-25 fractions.

\textit{Staining.} The silver staining method performed by Davis and Vincent\textsuperscript{8} was not
effective here in visualizing the isolated peptides, nor was staining with Coomassie Blue
effective. The only method which was able to visualize the peptides was pre-staining
with fluorescamine.

\textit{Molecular Weight.} The cobalt peptides in Co 4 and Co 5 samples have molecular
weights between 3500 and 4500. The literature value for the molecular weight of
LMWCr is 1500,\textsuperscript{8} implying that the isolated peptide is either not the cobalt-substituted
version of the one that Davis and Vincent isolated, or that it has been modified in such a
way as to increase its size.

Of course, the vitamin B\textsubscript{12}, and bacitracin standards seem to indicate that there
may be a large margin of error for mass determinations of low molecular weight
substances. This makes it possible that some of the difference between anticipated and
experimental masses is due to this margin of error, although it is dubious that the margin
is large enough to bring the MW of the sample in this study down to 1500.

The chromium peptides in Cr 1 and Cr 2 have MWs between 4500 and 5700, and
between 3900 and 4500, respectively. The MW determinations, particularly the SDS-
PAGE data, suggest that the peptide has the same MW as the Co peptide, suggesting that
the Cr and Co isolations may have isolated the same substance with different
substitutions. The HPLC data suggests, however, that there is some difference in the
MWs of the Co samples and in Cr 1, although this may be due to the broadness of the Cr 1
peak caused by the high concentration of the Cr 1 peptide.

Even if Cr 1 and Co 4 have differences other than their bound metals, it is
possible that they have the same amino acid sequence, but covalent modifications such as
glycosylation were made on one peptide (probably Cr 1, the larger peptide) but not the
other. It is also possible that either an impurity or the high concentration of peptide in the
Cr 1 fractions has distorted the peak.

The literature value for the molecular mass of LMWCr is reported as 1500 Da,\textsuperscript{8}
implying that the isolated peptides are not the same as that isolated by Davis and Vincent.
Of course, the vitamin B\textsubscript{12}, and bacitracin standards seem to indicate that there may be a
large margin of error for mass determinations of low molecular weight substances. This
makes it possible that some of the difference between anticipated and experimental
masses is due to this margin of error, although it is dubious that the margin is large
enough to bring the MW of the sample in this study down to 1500.
On the other hand, the data from the amino acid analysis suggests the amino acid portion of the peptides to have a molecular weight between 1000 and 1100 for Cr 1 and between 1000 and 1350 for Co 4. Both of these are quite close to the value obtained by estimating MW from the peptide content determined in Davis and Vincent (1100 using their integral estimates, 1200 using their actual data [Table 3]). This is consistent with the hypothesis that LMWCr is a multi-subunit peptide, and suggests that Davis and Vincent may have separated the subunits when preparing their peptide for SDS-PAGE.

**Metal-to-Peptide Ratio.** The metal-ion-to-free-amine ratio, which is assumed to be equivalent to the metal-to-peptide ratio, was between 3.3 and 3.7 for all Co 4, Cr 1, and Cr 2 although for Co 4. This is in agreement with the literature value of 3.5 obtained by Davis and Vincent, who also used the fluorescamine method to determine free amine concentration. Overall, the metal-to-peptide ratio is consistent with the hypothesis that Cr 1 and Cr 2 contain the peptide isolated by Davis and Vincent.

**Amino Acid Content.** Cr 1 in general has higher concentrations of amino acids that are not predicted to be in LMWCr than does Co 4. Assuming that the two samples contain the same peptide, the explanation may have to do with whatever is causing the shoulder and abnormally broad peak that were present on LMWCr HPLC chromatograms (Figure 13A). It may be necessary to further purify these samples or to do another isolation where the pooling of fractions is done more carefully.

Neither peptide had the same amino acid content as that isolated by Davis and Vincent. It is possible that much of this is due to contamination of the samples, as other evidence is supportive of the hypothesis that the isolated peptides are the same as the peptide isolated by Davis and Vincent.

**Summary.** The substances isolated in Cr 1 and Co 4 appear to be the same peptide bound to different metals, and moreover, appear to be the same material as isolated by Davis and Vincent. SDS-PAGE shows Co 4 and Cr 1 to contain substances of approximately the same molecular weight. Furthermore, when heated another band appears at a point which indicates the same MW as that which Davis and Vincent found for LMWCr, suggesting that individual LMWCr peptides may be part of a multi-subunit peptide. HPLC data suggests different MWs for the two peptides, but this may be due to an impurity or an excess of peptide in Cr 1 throwing off the peak location. Cr 2 appears at about the same MW as Co 4 and Co 5 on HPLC. In neither case is the MW that of LMWCr (according to Davis and Vincent), but this is consistent with LMWCr being a subunit of a larger peptide. In any case, the MWs obtained from HPLC and SDS-PAGE
are relatively close for Co 4, and Cr 2 (3900 from HPLC, 4500 from SDS-PAGE), and are only slightly further away for Co 5 and Cr 1 (3500 and 5700 from HPLC, respectively, and 4500 from SDS-PAGE).

Co 4, Cr 1, and Cr 2 all have metal-to-peptide ratios between 3.3 and 3.7, which are very close to the literature value of 3.5. The electronic spectrum of Cr 1 is almost identical to that of LMWCr in the literature. Moreover, amino acid analysis indicates that for both Cr 1 and Co 4, the most prevalent amino acids are those found in LMWCr according to Davis and Vincent, although not in the same ratios. Overall, the evidence points to the conclusion that the cobalt and chromium isolations contain the same peptide bound to different metals, and that they are the same as the peptide isolated by Davis and Vincent.
Conclusions

The data suggest that the Co 4 sample contains a protein which binds to about 4 cobalt ions and has a MW between 3900 and 4500. It contains significant quantities of cystenyl, glycyl, glutamyl, and asparryl residues. Whether or not this is LMWCo is uncertain, although some of the evidence (MW, high [cys]) would appear to be against it. The evidence also suggests that Cr 1 and Cr 2 contain a peptide or peptides that bind to about four chromium ions and which has a molecular weight between 3800 and 5800. Electronic spectrum analysis has shown great similarity between these two samples and the literature spectrum for LMWCr. In addition, it appears that the G-25 and G-15 columns do not provide a significant increase in the purity of the A-25 extract. Ultrafiltration is more effective at desalting samples than is dialysis. Of the many staining procedures attempted, fluorescamine is the only effective way to visualize the isolated peptides on an SDS-PAGE gel (which casts doubt on Davis and Vincent’s SDS-PAGE results, which were visualized using silver staining).8

In order to determine with more certainty the nature of the peptides which have been isolated, several things need to be done. More amino acid analyses need to be performed, second, it might be useful to perform SDS-PAGE and HPLC with a different set of standards in order to confirm the MW determinations. HPLC and SDS-PAGE on heated samples may also shed some light on whether previous MW results are those of single peptides or of complexes. It may be necessary to perform further purification on the peptide samples in order to make sure that they are not mixed with other peptides of similar MW. Finally, activity assays using the Cr peptide would be very helpful in determining whether or not LMWCr has been isolated.

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References


