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PREPARATIVE PURIFICATION OF *TRICHODERMA REESEI* NATIVE AND "CORE" CELLOBIOHYDROLASE I BY ELECTROPHORESIS AND CHROMATOFOCUSSING

Scientific Note

David A. Offord*, Norman E. Lee, Jonathan Woodward**

Chemical Technology Division
Oak Ridge National Laboratory***
Oak Ridge, TN 37831-6194

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*Oak Ridge Science and Engineering Research Semester Student, Bethany College, West Virginia.

**To whom correspondence should be addressed

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INTRODUCTION

The enzymes present in the cellulase complex produced by the fungus *Trichoderma reesei* have been the subject of considerable attention due to their potential for converting cellulosic materials into glucose for further use in fermentation processes [1]. Cellobiohydrolase I (CBH I) is the major component of crude commercial fungal cellulase preparations and catalyzes the conversion of insoluble cellulose into cellobiose. The primary structure of CBH I is known [2], and its tertiary structure, from small-angle x-ray scattering studies, takes the shape of a tadpole [3] with a catalytic head region known as "core" CBH I, and a C-terminal cellulose binding tail region. Removal of the tail can be accomplished with the protease papain, resulting in reduced activity towards insoluble substrates but with unchanged activity towards soluble substrates [4,5].

CBH I possesses 12 disulfide bonds [6], two of which reside in the C-terminal region [7]. We have been interested, recently, in the reduction of native and "core" CBH I and have needed to develop a method for their preparative purification. We now report that by using electrophoresis and chromatofocussing, preparative quantities of both native and "core" CBH I have been obtained. Since their pI values are different, milligram quantities of "core" CBH I can be generated and purified from the native enzyme by chromatofocussing within 2 h.

MATERIALS AND METHODS

Purification of Native CBH I

Preliminary Gel Filtration

Four mL of a crude cellulase preparation (Celluclast 250S, NOVO Enzymes, Danbury, CT) was initially separated by gel filtration on a 2.54 x 94-cm BioGel P-100 column. The fractions containing ρ-nitrophenyl cellobiosidase (PNPCase) activity measured as described in [8] were pooled, lyophilized, and reconstituted in nanopure water (5 mL), and the procedure was repeated.

Preparative Native Gel Electrophoresis

The filtered enzyme (4 mL, 17 mg/mL protein) was loaded into a single well (5 mm x 125 mm) and electrophoresis (Hoeffer Scientific, San Francisco, CA) was performed vertically on a 7.5% native polyacrylamide gel (3.75 mm x 13 cm x 14 cm) at 500 V and 150 mA until the dye marker (0.1% bromophenol blue) had migrated to the bottom of the gel. The band containing CBH I (located by coomassie blue staining of a gel run in parallel; see Fig. 1) was extracted from the gel by maceration in water, followed by centrifugation. After filtering and lyophilization, the enzyme was dissolved in 50 mM sodium acetate buffer, pH 5.0. The enzyme (5.2 mL, 22.5 mg) was then subjected to chromatofocussing using Fast Protein Liquid Chromatography (FPLC).

Fast Protein Liquid Chromatography (FPLC)

An enzyme sample obtained by electrophoresis (0.5 mL, 4.33 mg/mL) was further purified by chromatofocussing on an FPLC equipped with a Mono P HR 5/20 column and a UV detector (Pharmacia). A pH gradient was obtained by converting from a 100% 0.025 M methylpiperazine/HCl buffer (pH 5.5) to 100% of a 1:10 dilution of Polybuffer 74/HCl (pH 3.0) over a period of 45 min. Fractions (0.5 ml) were collected and those containing the major peak of protein (107-109; see Fig. 2a) were pooled and adjusted to pH 5.0 and rechromatofocussed (Fig. 2b). The fractions containing pure CBH I (105-109) were pooled and filtered through a PD-10 column with Sephadex G-25 M gel (Pharmacia) equilibrated with 50 mM sodium acetate buffer pH 5.0.

Purification of "Core" CBH I

Papain solution (32.8 µL, 2.46 mg/mL, 2 mM EDTA, 0.2 M NaH_2PO_4, 5 mM L-cysteine, pH 7.0) was incubated with 1 mL CBH I (2.46 mg/mL), at 37° C for 30 min according to the method of Abuja et al. [3]. The "core" CBH I was purified by chromatofocussing using the FPLC as described above. After 30 min of papain treatment, conversion was complete. A partial digest was obtained by omitting cysteine from the incubation medium.

Analytical Procedures

Gel electrophoresis of various enzyme fractions was carried out by standard procedures using equipment obtained from Hoeffer Scientific Instruments, San Francisco, or by using the Phastsystem (Pharmacia). Purified native and "core" CBH I were also checked for homogeneity by High Performance Liquid Chromatography (HPLC) using a Hewlett Packard HP 1090 Liquid Chromatograph equipped with a 75 x 7.5 mm Bio-Gel TSK-DEAE-5W anion exchange column (Bio-Rad) to determine its purity. The mobile phase consisted of 100% 50 mM sodium acetate buffer converted to 100% 0.5 M NaCl, 50 mM sodium acetate buffer pH 5.0 over 12 min, and elution continued until the 15 min mark with a flow rate of 1 mL/min. Detection was accomplished using a UV diode detector in a range from 260 nm to 300 nm with a maximum absorbance occurring at 280 nm. Plots of absorbance against wavelength and time presenting the data in three-dimensional form were achieved using the HP 1040. A data evaluation pack II (EVALU2) interfaced with the HPLC.

RESULTS AND DISCUSSION

Native and "core" CBH I were purified from a commercial preparation of cellulase in milligram quantities (Table 1). The low yields refer only to the total PNPCase activity and not to the actual yield of CBH I. PNPC is a substrate for both endoglucanase and β-glucosidase which cleave the aglycon bond in this substrate [9]. After preparative gel electrophoresis, the enzyme possessed neither endoglucanase (CMC viscosity reducing) nor β-glucosidase (cellobiase) activities indicating that these components had been removed. Based upon the CBH I activity obtained after preparative gel electrophoresis, the yield of activity of the pure enzyme after the second chromatofocussing step was about 23%.

Analytical SDS gel electrophoresis of CBH I after preparative gel electrophoresis showed it to be apparently homogeneous (Fig. 3); however, when subjected to chromatofocussing using FPLC, this was clearly not the case (Fig. 2a). The CBH I preparation consisted of at least five different

proteins whose pI values varied between pI 3.5 - 3.7 (see also Fig 4, lane 3). After rechromatofocussing fractions 107-109, one peak of protein was obtained (Fig. 2b) that was at least 99% homogeneous as shown by IEF (Fig. 4, lane 6). This was confirmed by a densitometric analysis of the IEF gel (data not shown). From Fig. 2b, the pI of CBH I was calculated to 3.61 in agreement with Hayn and Esterbauer [10]. CBH I eluted after 9.1 min using analytical HPLC and was also >99% homogeneous (Fig. 5). The specific activity of pure CBH I was 42% of that obtained by preparative gel electrophoresis, suggesting that chromatofocussing of CBH I leads to a loss in catalytic activity.

Treatment of pure CBH I with papain resulted in its complete conversion to "core" CBH I within 30 min as judged by IEF (Fig. 4, lane 7). Chromatofocussing by FPLC of a partial digest of native CBH I indicated this technique to be useful for the separation and purification of "core" CBH I whose pI was determined to be 3.23 (Fig. 6). After purification, the "core" was reconstituted in 50 mM sodium acetate buffer pH 5.0 and its homogeneity checked by HPLC. Elution occurred after 8.9 min and the "core" was >99% pure (data not shown). The finding that "core" CBH I was more acidic than native CBH I is in agreement with Van Tilbeurgh et al. (5). The specific activity of "core" CBH I with respect to PNPC hydrolysis was identical to the native enzyme.

The molecular size of native and "core" CBH I was analyzed by SDS electrophoresis using the Phastsystem (Fig. 7). The "core" enzyme is, as expected, smaller than the native enzyme. The reduced enzymes (samples boiled with mercaptoethanol prior to electrophoresis) were larger than the non-reduced samples, because presumably, the latter are more streamline and move faster through the gel. The estimated molecular weights for both the reduced and nonreduced enzymes are given in Table 2.

The nature of the minor bands observed during chromatofocussing of CBH I (after preparative gel electrophoresis) is not known. They could be subforms of CBH I differing in their levels of glycosylation [11]. Treatment of these proteins with papain did not result in the generation

of "core" CBH I so they are clearly not identical with pure native CBH I. Glycosylation could also be responsible for the stability of these forms against proteolysis by papain [12].

The results of this study show that extremely pure native and "core" CBH I can be obtained quickly and in large enough quantities to allow more accurate studies on their structure and properties. In this regard, "core" CBH II, but not its native form containing the glycosylated C-terminal region, has been crystallized [3].

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Table 1

Summary of the purification of native and "core" CBH I

Fraction	Volume (mL)	Protein (mg)	Activity (munits)	Activity (munits/mg)	Yield (%)
Gel Filtration	4.0	68.0	2638	38.8	100
Electrophoresis	5.2	22.5	448	19.9	17
FPLC (2nd run) (native CBH I)	5.0	12.3	102	8.4	4
"Core" CBH I	4.9	4.3	36	8.4	1.4

Table 2

Estimated molecular weight of reduced and nonreduced native and "core" CBH I*

	Native CBH I	"Core" CBH I
Reduced	67,000	55,000
Nonreduced	47,000	36,000

*Samples boiled with 2.5% SDS ± 5% β-mercaptethanol prior to electrophoresis.

LEGENDS TO FIGURES

Fig. 1 Preparative gel electrophoresis of crude gel-filtered cellulase. For details, see Materials and Methods.

Fig. 2. Chromatofocussing of electrophoretically prepared native CBH I. For details, see Materials and Methods. A: first chromatofocussing indicating heterogeneity of preparation; B: second chromatofocussing indicating homogeneity.

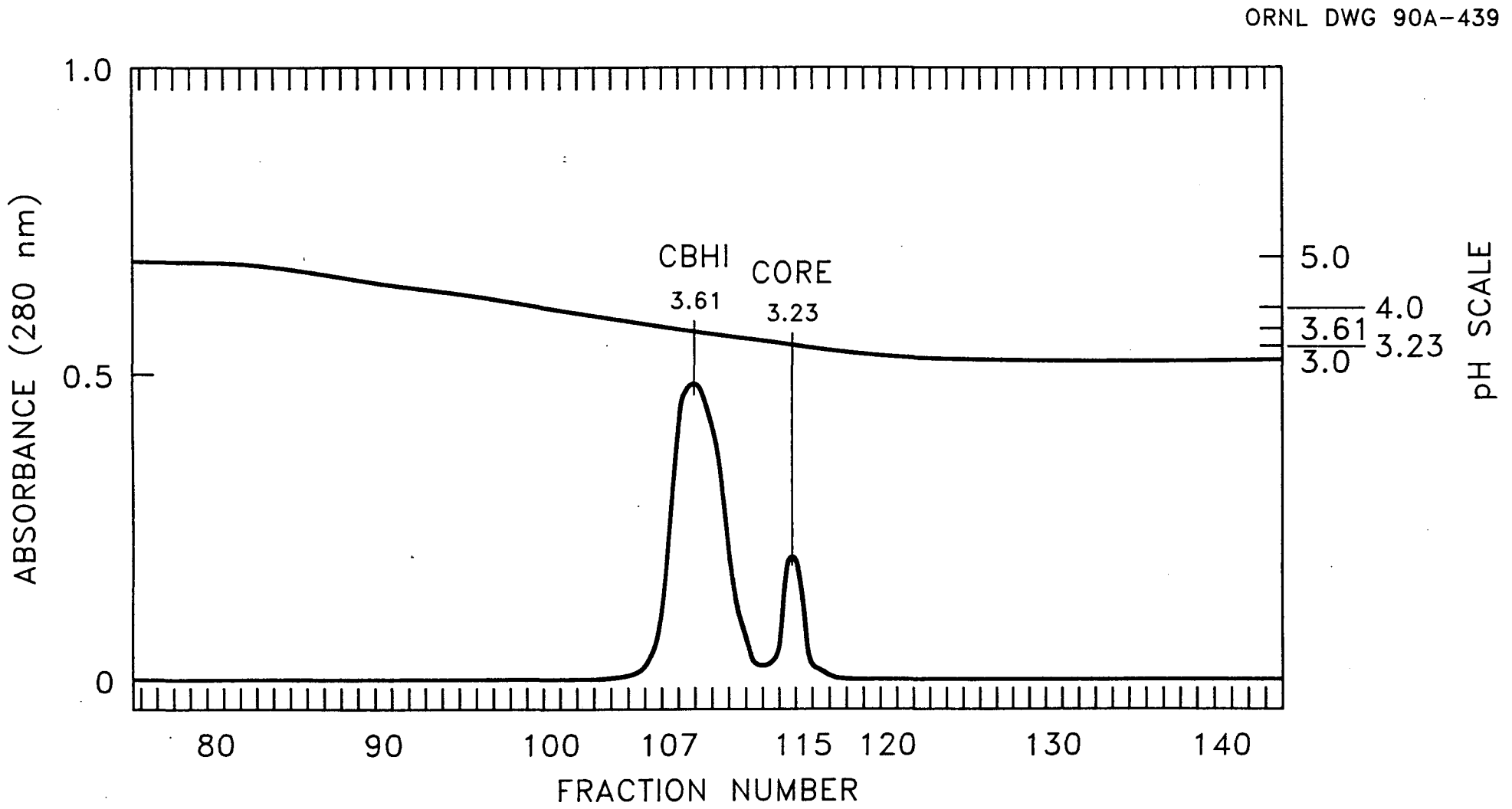
Fig. 3 SDS gel electrophoresis of native CBH I obtained after preparative gel electrophoresis. The electrophoretic system used was that obtained from Hoeffer, and the samples were reduced prior to electrophoresis. Acrylamide gels were 7.5%.

Fig. 4 Analytical isoelectric focussing (pH 3 to 9) of CBH I using the Phastsystem. Lanes 1, 4, 5, 8: pI markers; Lane 2: crude-filtered cellulase; Lane 3: CBH I after preparative gel electrophoresis; Lane 6, native CBH I after second chromatofocussing by FPLC; Lane 7, "core" CBH I purified by chromatofocussing. One-μL samples (1 to 3 μ g protein were applied to the gels).

Fig. 5 Analytical HPLC of CBH I. For details, see Materials and Methods. The peak of absorbance represents 47 mA units.

Fig. 6 Chromatofocussing by FPLC of a partial papain digest of native CBH I. For details, see Materials and Methods.

Fig. 7 SDS gel electrophoresis of native and "core" CBH I by the Phastsystem. Polyacrylamide gels (10 to 15%) were used in the analysis. Lane 1: molecular weight markers; Lane 2: "core" CBH I not reduced; Lane 3: "core" CBH I reduced; Lane 4: empty; Lane 5: native CBH I not reduced; Lane 6: native CBH I reduced; Lane 7: empty; Lane 8: markers (1 to 3 μg protein applied to the gels).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CORE
3.23
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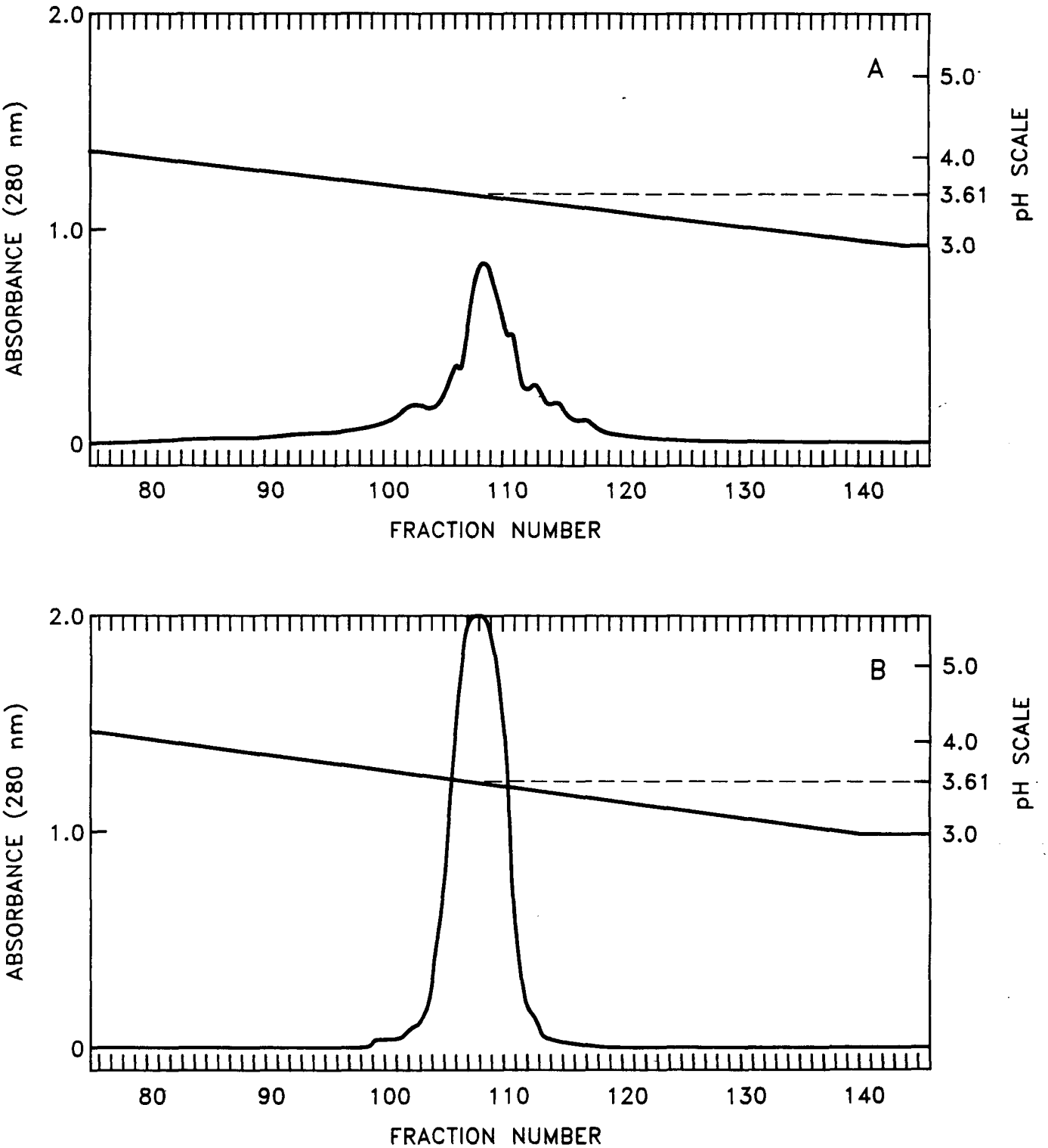

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