Purification of Eukaryotic RNA Polymerase II by Immunoaffinity Chromatography

ELUTION OF ACTIVE ENZYME WITH PROTEIN STABILIZING AGENTS FROM A POLYOL-RESPONSIVE MONOCLONAL ANTIBODY*

(Received for publication, October 16, 1989)

Nancy E. Thompson, Dallas B. Aronson, and Richard R. Burgess
From the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Active eukaryotic RNA polymerase II (RNAP II) was purified by immunoaffinity chromatography, using a monoclonal antibody (mAb) that reacts with the highly conserved heptapeptide repeat of the largest subunit. This mAb (designated 8WG16) was conjugated to CNBr-activated Sepharose and used to purify RNAP II from wheat germ and calf thymus. The subunit composition of the immunoaffinity-purified enzyme was essentially the same as RNAP II purified by conventional chromatography except that it contained only the form with the unproteolyzed largest subunit. Active enzyme could be eluted from the 8WG16-Sepharose, at pH 7.9, with combinations of low molecular weight polyols and nonchaotropic salts. The superior eluting procedure used combinations of ethylene glycol (30-40%) and ammonium sulfate (0.5-0.75 M). Active enzyme also could be eluted with a synthetic peptide containing four repeats of the heptapeptide; however, the peptide was not as effective as the polyol and salt combinations for eluting the enzyme. This mAb should be useful for purifying RNAP II from many eukaryotic species.

Because the elution of enzyme from the immunoafford-sorbent seems to be dependent upon the presence of a polyol, this antibody is referred to as a "polyol-responsive mAb." A procedure that helps to identify a polyol-responsive mAb and to optimize the eluting conditions is described. Polyol-responsive mAbs might have broad applicability to the purification of many labile enzymes by immunoaffinity chromatography.

Little is known about the role that each subunit plays in either the catalytic or regulatory activity of the enzyme. The properties of RNAP II from various sources and the comparison of their subunit structures with RNA polymerases I and III have been reviewed (1-3). Regulation of RNAP II by transcription factors has been recently reviewed (4-7).

RNAP II has been difficult to purify from almost any source. Most procedures require the use of several chromatographic and concentration steps. These steps often result in low yields and reduced specific activity. The use of polyethyleneimine (Polymin P) to precipitate RNAP II from dilute solution as described by Jendrisak and Burgess (8) has greatly simplified the purification and has been incorporated into procedures for the large scale purification of RNAP II from many sources (9-12).

Interpretation of the early literature on RNAP II was complicated by the presence of subforms of RNAP II that differ in the size of the largest subunit (approximately 240 versus 220 versus 180). Subsequent studies established that the 180-kDa subunit results from proteolysis of the 220-kDa subunit during the purification process (12-14). The 240-kDa subunit probably results from the phosphorylation of the 220-kDa subunit (15, 16). In mammalian cells, the form of RNAP II that contains the phosphorylated largest subunit (240 kDa) is sometimes referred to as II0, the form containing the nonphosphorylated, unproteolyzed largest subunit (220 kDa) as II, and the form containing the proteolyzed largest subunit (180 kDa) as IIB (17). Recently, Kim and Dahmus (18) have reported a procedure by which calf thymus RNAP II can be separated into forms II0, IIA, and IIB.

Cloning and sequencing studies have revealed that the largest subunit of RNAP II from yeast (19), mouse (20), hamster (21), Drosophila melanogaster (21, 22), and Caenorhabditis elegans (23) contains an unusual heptapeptide repeat on the C-terminal end. The consensus sequence for this heptapeptide is Pro-Thr-Ser-Pro-Ser-Tyr-Ser. The number of repeats varies from 26 in yeast (19) to 52 in the mouse (20). Deletion analyses have revealed that approximately one half of the repeats are necessary for cell viability (21, 22, 24, 25).

Proteolysis of the 220-kDa subunit releases the heptapeptide-containing domain, generating the 180-kDa subunit (16, 19, 20). In addition, phosphorylation of this domain probably converts the apparent molecular weight of the largest subunit to 240 kDa (16).

We have reported the isolation of several monoclonal antibodies (mAbs), raised against wheat germ RNAP II, that react with the conserved heptapeptide repeat (26). We report here the development of an immunoaffinity chromatography procedure for the purification of RNAP II that uses one of these mAbs. This procedure affords two distinct advantages.

Eukaryotic RNA polymerase II (RNAP II)1 synthesizes the precursors to messenger RNAs and the small nuclear RNAs with the exception of U6 small nuclear RNA. This multimeric enzyme can consist of up to 12 subunits depending upon the species from which it is isolated. The basic subunit structure consists of two large subunits (approximately 220 and 140 kDa) and six to ten smaller subunits (less than 100 kDa).

This research was supported by Grants CA07175 and CA23076 from the National Cancer Institute and Grant GM28575 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

1 The abbreviations used are: RNAP II, RNA polymerase II; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TE, Tris-EDTA buffer.
over most other methods for purifying RNAP II. First, because the heptapeptide repeat is highly conserved, a single mAb can be used to isolate RNAP II from many sources. Second, because the mAb reacts with the heptapeptide repeat, only RNAP II containing the unproteolyzed largest subunit is isolated. In addition, a novel method for eluting active RNAP II from the immunoabsorbent is described. This elution method, which uses low molecular weight polyhydroxylated compounds (polyols) in combination with salt, can be performed at close to physiological pH values. Therefore, this elution method might have applicability to the purification of other enzymes by immunoaffinity chromatography.

MATERIALS AND METHODS AND RESULTS

Characteristics of mAb 8WG16—When wheat germ RNAP II is used as an immunogen, the majority of the mAbs produced by the hybridomas react with the heptapeptide repeat but are IgM molecules (26). However, antibody 8WG16 is an IgG2a mAb that reacts with this domain. The SDS-polyacrylamide gel in Fig. 1 (lanes 3 and 4) shows the two largest subunits (220 and 140 kDa) of wheat germ and calf thymus RNAP II. Most of the largest subunit from wheat germ is in the unproteolyzed form (220 kDa); most of the largest subunit of the calf thymus enzyme is in the proteolyzed form (180 kDa). The corresponding immunoblot in Fig. 1 shows that antibody 8WG16 reacts with the unproteolyzed largest subunit (220 kDa) of calf thymus RNAP II (lane 1), mAb 8WG16 also gives a strong reaction with the unproteolyzed subunit (220 kDa) of calf thymus RNAP II (lane 2), although this form of the subunit is present in an insignificant amount. mAb 8WG16 also reacts with some species of molecular mass lower than 220 kDa in the calf thymus preparation; however, we believe these bands to be products of partial proteolysis.

The reactivity of mAb 8WG16 with the synthetic peptide containing four repeats of the heptapeptide repeat is described in Fig. 2.

Like other mAbs that react with heptapeptide repeat, 8WG16 inhibits promoter-directed transcription, but it does not inhibit elongation in the nonspecific transcription assay (26, 27).

Use of Polyols for Eluting RNAP II—A screening procedure (designated ELISA-elution assay) described under "Materials and Methods" and Fig. 3 helped to establish non-denaturing conditions that would result in the disruption of the antigen-antibody complex. Results obtained from this assay indicated that either ethylene glycol or glycerol (polyols), alone or in combination with salt, disrupted the antigen-antibody complex. Although several salts, in combination with a polyol, seemed to be effective at disrupting the antigen-antibody complex (Fig. 3A), ammonium sulfate was chosen because of its protein stabilizing properties.

We tested the ability of polyols, alone and in combination with ammonium sulfate, to elute RNAP II from mAb 8WG16. Wheat germ was processed as described under "Materials and Methods." 8WG16-Sepharose, to which the wheat germ RNAP II had been adsorbed, was washed extensively with TE containing 200 mM ammonium sulfate and then eluted with TE containing either 50% ethylene glycol, 50% glycerol, 50% ethylene glycol and 1 M ammonium sulfate, or 50% glycerol and 1 M ammonium sulfate. Enzyme was eluted by a batch method using 3 ml of eluting reagent/2 ml of packed Sepharose for 20 min at room temperature. The Sepharose was immediately eluted a second time with 3 ml of the same reagent for 20 min at room temperature. Each sample of 8WG16-Sepharose was then washed with 3 ml of 2 M KSCN to remove the remaining RNAP II. All samples were dialyzed overnight against storage buffer at 4 °C. Samples were assayed for protein concentration and enzyme activity; purity was assessed by SDS-PAGE. The enzyme recovered by the immunoaffinity method was compared for activity and purity to enzyme that was prepared from the same lot of wheat germ by the conventional chromatographic method.

Enzyme with high specific activity was recovered by all four methods of elution (Table I). However, recovery of enzyme was highest with TE containing 50% ethylene glycol and 1 M ammonium sulfate. The SDS-polyacrylamide gel in Fig. 4 shows the purity of the enzyme recovered by ethylene glycol or glycerol in combination with ammonium sulfate. Propylene glycol and 2,3-butanediol were found to be as effective as...
Elution of Enzyme by Peptide—The ability of the synthetic ethylene glycol when combined with ammonium sulfate (data not shown).

Elution with Ethylene Glycol Containing Ammonium Sulfate—Because we found that the enzyme activity was variable when 50% ethylene glycol was used, we examined varying concentrations of ethylene glycol and ammonium sulfate to determine the lowest concentration of each reagent that would effectively disrupt the antigen-antibody complex and retain enzyme activity. Results from the stability studies and ELISA-elution assay are shown in Figs. 5 and 6, respectively.

The efficacy of lower concentrations of ethylene glycol and ammonium sulfate to elute RNAP II from 8WG16-Sepharose was examined. Wheat germ was processed for a large-scale RNAP II preparation. Approximately 3 ml of 8WG16-Sepharose was eluted sequentially with TE containing 30% ethylene glycol and 0.5 M ammonium sulfate. Another 3 ml of Sepharose was eluted sequentially with TE containing 40% ethylene glycol and 0.75 M ammonium sulfate. Each 8WG16-Sepharose was then washed with 2 M KSCN. All elutions were then dialyzed against storage buffer.

The recovery of enzyme when these combinations were used in four sequential elutions is shown in Table II. The SDS-polyacrylamide gel in Fig. 7 shows the purity of the enzyme recovered when the 8WG16-Sepharose was eluted with TE containing 30% ethylene glycol and 0.5 M ammonium sulfate. The majority of the wheat germ RNAP II could be removed from the 8WG16-Sepharose by the four sequential elutions of ethylene glycol and ammonium sulfate. All of these elutions showed specific activity at least as high as the enzyme prepared by conventional chromatography. Table II also compares the recovery of enzyme prepared by the immunoaffinity method with enzyme prepared by conventional chromatography from the same lot of wheat germ. Comparison of the total activity of the material before and after the immunoaffinity step established that the 8WG16-Sepharose adsorbed approximately 75% of the RNAP II activity during the 2 h of exposure in the batch method.

Elution of Enzyme by Peptide—The ability of the synthetic ethylene glycol when combined with ammonium sulfate (data not shown).

Elution with Ethylene Glycol Containing Ammonium Sulfate—Because we found that the enzyme activity was variable when 50% ethylene glycol was used, we examined varying concentrations of ethylene glycol and ammonium sulfate to determine the lowest concentration of each reagent that would effectively disrupt the antigen-antibody complex and retain enzyme activity. Results from the stability studies and ELISA-elution assay are shown in Figs. 5 and 6, respectively.

The efficacy of lower concentrations of ethylene glycol and ammonium sulfate to elute RNAP II from 8WG16-Sepharose was examined. Wheat germ was processed for a large-scale RNAP II preparation. Approximately 3 ml of 8WG16-Sepharose was eluted sequentially with TE containing 30% ethylene glycol and 0.5 M ammonium sulfate. Another 3 ml of Sepharose was eluted sequentially with TE containing 40% ethylene glycol and 0.75 M ammonium sulfate. Each 8WG16-Sepharose was then washed with 2 M KSCN. All elutions were then dialyzed against storage buffer.

The recovery of enzyme when these combinations were used in four sequential elutions is shown in Table II. The SDS-polyacrylamide gel in Fig. 7 shows the purity of the enzyme recovered when the 8WG16-Sepharose was eluted with TE containing 30% ethylene glycol and 0.5 M ammonium sulfate. The majority of the wheat germ RNAP II could be removed from the 8WG16-Sepharose by the four sequential elutions of ethylene glycol and ammonium sulfate. All of these elutions showed specific activity at least as high as the enzyme prepared by conventional chromatography. Table II also compares the recovery of enzyme prepared by the immunoaffinity method with enzyme prepared by conventional chromatography from the same lot of wheat germ. Comparison of the total activity of the material before and after the immunoaffinity step established that the 8WG16-Sepharose adsorbed approximately 75% of the RNAP II activity during the 2 h of exposure in the batch method.
Immunoaffinity Chromatography of RNA Polymerase II

Fig. 9. SDS-PAGE of wheat germ RNAP II eluted from 8WG16-Sepharose by the synthetic peptide. Lanes 1 and 2 contained material (2.6 and 0.3 μg) that was eluted by two sequential applications of 400 μg peptide/ml; lane 3 contained material (5.0 μg) that was eluted with the 2 M KSCN wash of this 8WG16-Sepharose. Lane 4 contained 2 μg of wheat germ RNAP II that was purified by conventional chromatography.

Fig. 10. SDS-PAGE of RNAP II that was purified from calf thymus using the immunoaffinity procedure. Lane 1 contained 5 μl of the starting material for the immunoaffinity chromatography step. Lanes 2–5 contained material (0.3, 1.4, and 1.6 μg) that was eluted by three sequential elutions of 30% ethylene glycol containing 0.5 M ammonium sulfate. Lane 5 contained material (4.2 μg) that was eluted with the 2 M KSCN wash of the 8WG16-Sepharose. Lane 6 contained 2 μg of calf thymus RNAP II that was purified by the conventional chromatographic procedure of Hodo and Blatti (9). Polyptides of approximately 40 and 50 kDa in the conventionally purified material are contaminants of this particular preparation. Lane 7 contained molecular mass markers.

The ability of the synthetic peptide to elute RNAP II from 8WG16-Sepharose was also tested. Wheat germ was processed as for large scale RNAP II preparation. Approximately 3 ml of 8WG16-Sepharose to which the RNAP II was adsorbed was eluted twice with 3 ml of TE containing 400 μg synthetic peptide/ml for 20 min at room temperature. The eluted proteins were dialyzed against storage buffer and then assayed for purity (Fig. 9) and activity. Some enzyme (approximately 0.38 mg) with relatively high specific activity (379 units/mg) could be eluted from the column with the first elution. However, the second elution did not yield a significant amount of enzyme (Fig. 9).

Purification of RNAP II from Calf Thymus—To test the ability of this method to purify RNAP II from another eukaryotic organism, we examined calf thymus. Calf thymus RNAP II was processed as described under “Materials and Methods.” Because calf thymus RNAP II seems to be more labile than the wheat germ enzyme, the 8WG16-Sepharose was eluted with TE containing 30% ethylene glycol and 0.5 M ammonium sulfate for 20 min at room temperature. Like the wheat germ enzyme, the calf thymus enzyme could be recovered by three sequential elutions of these reagents (Fig. 10 and Table III).

Table III. Recovery of calf thymus RNAP II from 8WG16-Sepharose using sequential elutions of 30% ethylene glycol containing 0.5 M ammonium sulfate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>1175</td>
<td>247</td>
<td>0.21</td>
</tr>
<tr>
<td>Flow-through</td>
<td>1100</td>
<td>165</td>
<td>0.15</td>
</tr>
<tr>
<td>1st elution</td>
<td>0.08</td>
<td>5.8</td>
<td>70</td>
</tr>
<tr>
<td>2nd elution</td>
<td>0.125</td>
<td>10.3</td>
<td>82</td>
</tr>
<tr>
<td>3rd elution</td>
<td>0.145</td>
<td>12.2</td>
<td>84</td>
</tr>
<tr>
<td>KSCN wash</td>
<td>0.375</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a One unit of activity represents the incorporation of 1 nmol of UTP.

DISCUSSION

This paper describes a method for rapidly purifying active RNAP II by immunoaffinity chromatography. The elution conditions presented in this paper might have general applicability to the purification of other labile enzymes by immunoaffinity chromatography.

Properties of mAb 8WG16—The procedures described in this paper use a mAb that reacts with the unusual heptapeptide repeat found on the largest subunit of RNAP II. This domain has been highly conserved in eukaryotic organisms except for Trypanosoma species (33, 34). The advantages of using a mAb that reacts with the heptapeptide repeat are that a single antibody column can be used to isolate RNAP II from a variety of species, and the recovered enzyme contains only the form with the unproteolyzed largest subunit. Although RNAP II that lacks the C-terminal domain can transcribe from certain promoters in vitro (22, 26, 35), the form containing this unusual domain is likely the physiologically relevant form of the enzyme.

Although we have isolated many antibodies that react with the heptapeptide repeat, most of these antibodies have been IgM molecules (26, 27). Dahmus et al. (36) also reported an immunodominance of the heptapeptide repeat when calf thymus RNAP II is used directly as the immunogen. Using some of the methods described in this paper, we have had limited success purifying RNAP II using these IgM molecules (27). However, the IgM-conjugated Sepharose was not stable to repeated use. Thus, a new antibody column had to be prepared for each RNAP II preparation. In contrast, antibody 8WG16 seems to be highly stable, and the same antibody-conjugated Sepharose has been used at least six times without noticeable deterioration.
The synthetic peptide containing four repeats of the heptapeptide repeat reacts with antibody 8WG16 (Fig. 2). This peptide is different from the peptide reported in a previous paper (26) in that it contains four repeats instead of three, and it does not contain cysteine residues on the termini. Reactivity of 8WG16 with the new peptide could not be demonstrated directly in either the ELISA or the dot blot assay; we suspect that without the terminal cysteine residues the peptide does not adhere well to polystyrene or nitrocellulose. It is also possible that the cysteine-containing peptides might oxidize to larger polymers that adhere more efficiently to nitrocellulose or polystyrene. However, antibody 8WG16 reacts well in both of these assays when the new peptide was conjugated to BSA (Fig. 2, A and B). In addition, reaction with the peptide inhibits binding of 8WG16 to wheat germ RNAP II in the ELISA (Fig. 2C). Therefore, it is not surprising that the peptide can elute some wheat germ RNAP II from 8WG16-Sepharose (Fig. 9). However, sequential elution with the peptide was not very effective (Fig. 9). This indicates that some RNAP II molecules might be bound more tightly to the antibody. Enzyme that is bound by more than one antibody (due to the highly repetitive nature of the C-terminal domain) would probably be more difficult to elute by the peptide.

Purification of Wheat Germ RNAP II—Wheat germ RNAP II of high purity and high specific activity was obtained by eluting the immunosorbent with polyols containing ammonium sulfate. Enzyme purified by the immunoaffinity chromatography procedure was compared with RNAP II purified from the same lot of wheat germ by the conventional chromatographic method. The subunit composition of the immunoaffinity-purified enzyme is essentially the same as the subunit composition of the conventionally purified enzyme, except that the immunoaffinity procedure recovered only the form containing the unproteolyzed largest subunit (Figs. 4 and 7). The immunoaffinity-purified enzyme had slightly higher specific activity than enzyme prepared by the conventional method (Tables I and II). The immunoaffinity step resulted in a 100-fold purification of wheat germ RNAP II (Table II). About 75% of the enzyme activity adhered to the 8WG16-Sepharose during the 2 h of batch adsorption. We believe that the remaining 25% represents the proteolyzed form of the enzyme that does not adsorb to the antibody but is active in the nonspecific transcription assay (26, 27). However, the immunoaffinity method recovered the same amount of enzyme as the conventional method (approximately 3 mg/167 g of wheat germ). Although the actual capacity of the immunosorbent is not known, the theoretical capacity of 3 ml of 8WG16-Sepharose, prepared as described, as about 50 mg of wheat germ RNAP II. (This estimation assumes that all antibody is active and that one IgG can bind two molecules of RNAP II). Although the entire purification procedure is generally performed over two days, it can be accomplished in a single work day of approximately 16 h.

Purification of Calf Thymus RNAP II—Using a procedure similar to the wheat germ purification, we have been able to use the 8WG16-Sepharose to purify RNAP II from calf thymus (Fig. 10). Due to the large amount of protein and DNA in the crude material, the preparation of the calf thymus material before application to the immunoaffinity adsorbent involves more steps than the wheat germ procedure. The high proteolytic activity in calf thymus rapidly removes the C-terminal domain of the largest subunit (9), rendering a large proportion of the RNAP II unreactive with the antibody. The initial ammonium sulfate precipitation step, as described under “Materials and Methods,” was necessary to remove much of this proteolytic activity. Although the recovery of the calf thymus enzyme by the immunoaffinity procedure seems low, immunoblots established that most of the form containing the C-terminal domain is adsorbed by the 8WG16-Sepharose (data not shown). Thus, much of the activity in the starting material for the immunoaffinity step is due to the proteolyzed form of the enzyme, which does not adhere to the 8WG16-Sepharose. Furthermore, the calf thymus enzyme appears to be very labile in the purified form.

Specific Initiation with Immunoaffinity-purified RNAP II—Although the wheat germ enzyme shows high specific activity in the nonspecific transcription assay, the ability of this enzyme to initiate correctly is not known. An extract prepared from wheat germ by a procedure almost identical to the preparation of the crude material for immunoaffinity chromatography has been reported to accurately transcribe a plant promoter (37). We have prepared extracts according to this procedure and have examined several promoters, but, to date, we have not been able to document accurate transcription using these extracts. Because the wheat germ RNAP II does not substitute for the mammalian RNAP II in HeLa cell extracts (38), we have not been able to test the ability of the wheat germ enzyme prepared by immunoaffinity chromatography to accurately transcribe from a promoter. Previously, we showed that calf thymus RNAP II that was prepared by the immunoaffinity chromatography procedure has initiation activity in HeLa cell nuclear extracts (26). Finally, 8WG16-Sepharose has been used to purify RNAP II from yeast; the yeast enzyme is active in an initiation assay (39). Thus, with some modification, this immunoaffinity chromatography system is capable of purifying RNAP II from other eukaryotic sources, that the heptapeptide repeat is present.

Phosphorylation State—The immunoaffinity chromatography procedure purifies only the form of RNAP II containing the unproteolyzed largest subunit. However, the phosphorylation state of the enzyme is not known. Attempts to shift the size of the largest subunit of the calf thymus RNAP II by treatment with phosphatases or kinases have not been successful (data not shown). Although the phosphorylated form of the largest subunit is present in mammalian cells (16), it is not clear if phosphorylation is necessary for initiation or a consequence of the initiation process (40). Clearly, enzyme prepared by the immunoaffinity procedures shows initiation activity in vitro (26, 39).

Selection of Eluting Conditions—A homogeneous antibody population should respond uniformly to eluting reagents or conditions. Screening mAbs for the ability to release antigen in response to changes in pH value or salt concentration has been reported (41, 42).

RNAP II, like many other enzymes, is extremely sensitive to even moderate conditions that commonly are used to elute antigen from immunosorbents (chaotropic salts such as KSCN, changes in pH values, denaturing reagents such as urea or guanidine HCl). The formidable task of testing hundreds of conditions that seemed “gentle” was expedited by the use of the simple ELISA-elution screening assay. This procedure is similar to the one suggested by Cobbs (42) for low affinity antibodies; however, we feel that the use of polyols allows this screening assay to be used with high affinity antibodies. The advantage of this assay is that many reagents (Fig. 3A) or even subtle changes in reagent composition (Fig. 6) can be tested on one polystyrene plate. The ELISA-elution assay measures the elution of antibody from immobilized antigen; we have found that this system is not completely representative of the elution of antigen from immobilized antibody in an actual immunoaffinity chromatography pro-
 been purposefully addressed. The use of 50% ethylene glycol elution assay do not react with the antigen in solution.3 We and ammonium sulfate elute active enzyme from the antibody water structure makers (46-48). The effect of these reagents ethylene glycol should weaken hydrophobic interactions while conjunction of hydrophobic and electrostatic interactions; we recover more enzyme with sequential elutions resulted in good recovery of the enzyme. Ammonium sulfate is the preferred salt because of its protein stabilizing properties, although sodium chloride, sodium acetate, and potassium glutamate are also effective (data not shown). Although ethylene glycol or glycerol are commonly used in polyols and salt was an unexpected result. We have designated mAbs that respond to this type of elution procedure as “polyl-responsive mAbs.” This paper presents data using ethylene glycol or glycerol as the polyol; recently we have found that propylene glycol or 2,3-butanediol are as effective as ethylene glycol (data not shown). The addition of salt to the polyol greatly increases the recovery of the enzyme. Ammonium sulfate is the preferred salt because of its protein stabilizing properties, although sodium chloride, sodium acetate, and potassium glutamate are also effective (data not shown). Although ethylene glycol or glycerol are commonly used in conjunction with salt in some protein purification systems, the efficacy of these combinations as eluting reagents for recovery of enzymes from immunoaffinity adsorbents has not been purposefully addressed. The use of 50% ethylene glycol at high pH values (10.5-11.5) to elute immunoaffinity adsorbents has been documented (43, 44). Aerts et al. (45) were able to use pH 6.5 but had to increase the ethylene glycol concentration to 90% for a highly hydrophobic enzyme. We found that RNAP II is unstable in 50% ethylene glycol (Fig. 5), although stability might be influenced by how quickly the ethylene glycol is removed. The results presented in this paper indicate that polyols (30-40%) in combination with nonchao- tropic salt (0.5-0.75 M) allowed the recovery of active enzyme at close to physiological pH values. In addition, the ability to recover more enzyme with sequential elutions resulted in good recovery of the enzyme.

The mechanism by which combination of ethylene glycol and ammonium sulfate elute active enzyme from the antibody is not obvious. Most antibody-antigen interactions are a combination of hydrophobic and electrostatic interactions; we have no reason to believe that the interaction of antibody SWG16 with its epitope is any different. Theoretically, the ammonium sulfate should weaken electrostatic interactions while strengthening hydrophobic interactions; conversely, the ethylene glycol should weaken hydrophobic interactions while strengthening electrostatic interactions. Both ethylene glycol and sulfate ions have been described as polar kosmotropes or stabilization of the enzyme. How can a combination of two stabilizing agents result in the disruption of an antigen- antibody complex? Our tentative explanation for the behavior of this system is that the ammonium sulfate and ethylene glycol strengthen interactions within the enzyme itself, or without the antibody itself, which results in sterically desta- bilizing the antigen-antibody interactions.

We have examined several panels of mAbs prepared against other proteins for polyl responsiveness by the ELISA-elution assay. Generally, we have found that 10-20% of the mAbs seem to be sensitive to the salt/polyl combination. However, some mAbs that seem to respond to salt/polyl in the ELISA-elution assay do not react with the antigen in solution.2 We attribute this artifact to distortion of the antigen when it is immobilized on the polystyrene plate. In this case, treatment with the salt/polyl might relieve some of this distortion, resulting in essentially a “false-positive” ELISA-elution assay. This type of mAb is not useful for immunoaffinity chromatography.

In conclusion, we believe that a polyl-responsive elution procedure might allow the powerfully selective technique of immunoaffinity chromatography to be used for the purification of many proteins, especially labile enzymes that are inactivated by lengthy purification procedures.

REFERENCES


Immunoaffinity Chromatography of RNA Polymerase II


Subheading Material To.

PURIFICATION OF EUKARYOTIC RNA POLYMERASE II

BY IMMUNOAFFINITY CHROMATOGRAPHY

ELUTION OF ACTIVE ENZYMES BY PROTEIN LABELING WITH AN IMMUNOLOGICAL ANTIBODY

Nancy E. Thompson, Dallas B. Armstrong, and Richard H. Burgess

Materials and Methods
Buffers and Reagents

The isolation of the histidinyl peptide antibody GM16 was completed (28). Antibody GM16 was purified from culture fluids and precipitated by precipitation with 8-mercaptoethanol. After precipitation with 10% protein A-Sepharose (Pharmacia, Piscataway, NJ), the antibody was equilibrated in Chromobind buffer and dialyzed into Chromobind buffer. The antibody was stored as a suspension of 2 mg/ml in 0.1 M NaCl, 0.1 M sodium phosphate, 0.02% NaN3, 0.02% NaN3, and 0.05% NaN3, 0.02% NaN3, and 0.05% NaN3, 0.02% NaN3. Antibody GM16 was stored at 4°C in a nitrogen atmosphere.

Antibody GM16 was adsorbed with a rabbit anti-serum to a rabbit IgG fraction and cross-reacted with a synthetic histidinyl peptide (GM16). Antibody GM16 was prepared with the rabbit anti-serum to IgG fraction (28).

RNA Polymerase II

Antibody GM16 was purified by a standard chromatographic technique using a Sepharose-CL4B column (Pharmacia, Piscataway, NJ) with the modifications described by Aerts and Bruns (28) through the use of an affinity chromatography step. Briefly, the antibody (500 ml) was incubated in 2 ml of 20 mM Tris, pH 7.4, 0.1 M NaCl, and 0.1 M EDTA, and then precipitated with 10% protein A-Sepharose. The precipitated material was washed with 10 volumes of 10 mM Tris, pH 7.4, 0.1 M NaCl, 0.1 M EDTA, and 0.01% NaN3, 0.01% NaN3. Antibody GM16 was stored at 4°C in a nitrogen atmosphere.

Enzyme-Linked Immunoabsorbent Assays (ELISA) and ELISA Colloidal Gold

Antibody GM16 was used to purify standard chromatographic techniques were employed by the method of Laskowski and Burgess (30) with modifications described by Aerts and Bruns (28) through the use of an affinity chromatography step. Briefly, the antibody (500 ml) was incubated in 2 ml of 20 mM Tris, pH 7.4, 0.1 M NaCl, and 0.1 M EDTA, and then precipitated with 10% protein A-Sepharose. The precipitated material was washed with 10 volumes of 10 mM Tris, pH 7.4, 0.1 M NaCl, 0.1 M EDTA, and 0.01% NaN3, 0.01% NaN3. Antibody GM16 was stored at 4°C in a nitrogen atmosphere.

Antibody GM16 was adsorbed with a rabbit anti-serum to a rabbit IgG fraction and cross-reacted with a synthetic histidinyl peptide (GM16). Antibody GM16 was prepared with the rabbit anti-serum to IgG fraction (28).

Western blotting was performed according to the procedure for immunoblotting, except the 20-kDa antibody GM16 was detected by an avidin-biotin complex (1:10000 dilution) followed by peroxidase-labeled avidin and 3,3-diaminobenzidine chromogen. Bands were visualized by autoradiography (28).

Electrophoresis and Immunoblotting

Proteins were separated by electrophoresis according to the method of Laemmli (29) using a separation gel containing 15% (w/v) polyacrylamide and 0.1% SDS. Electrophoresis was performed on a Novex 5x8 cm acrylamide gel (Novex, San Diego, CA). After electrophoresis, gels were stained with 0.1% Coomassie Blue in 20% methanol and 10% acetic acid, then destained with 10% methanol and 10% acetic acid. The gels were dried and autoradiographed with an intensifying screen at -80°C for 2-4 h. The positions of the bands were determined by comparison with a standard of known molecular weight. The bands were excised and eluted in Tris-buffered saline (pH 8.0) or 1% SDS and stored at -20°C for subsequent analysis. The proteins were transferred to nitrocellulose by electroblotting, and the blots were probed with an antibody to GM16 using a Bio-Rad microinterior separator (Bio-Rad Laboratories, Richmond, CA).

Monospecific Transfection Assays

Recombinant proteins were produced in E. coli. The recombinant proteins were purified by affinity chromatography using the rabbit anti-serum to IgG fraction (28). The proteins were then eluted with a solution of 1 M NaCl, 0.1 M sodium phosphate, 0.02% NaN3, 0.02% NaN3, and 0.05% NaN3, 0.02% NaN3, and 0.05% NaN3, 0.02% NaN3.

Antibody GM16 was adsorbed with a rabbit anti-serum to a rabbit IgG fraction and cross-reacted with a synthetic histidinyl peptide (GM16). Antibody GM16 was prepared with the rabbit anti-serum to IgG fraction (28).

Results

Proteins were determined by the method of Bradford (30), using the Coomassie blue stain as a standard.

Protein Concentrations

Proteins were determined by the method of Bradford (30), using the Coomassie blue stain as a standard.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.

Results

Recipient plasmids were isolated from a yeast genomic library. The yeast genomic library was transformed into E. coli, and the transformants were screened by plasmid isolation. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing. The plasmids were then analyzed by restriction enzyme digestion and Southern blotting. The plasmids were then amplified and characterized by DNA sequencing.
Fig. 3. ELISA-Eulsion Assay Using Different Stabilizing Reagents. Fig. 3A. MA-8BWG2 was reacted with wheat germ RPNP II that was immobilized on the wells of a polystyrene plate. The antigen-antibody complex was then treated with TE (1), TE containing 1 M NaCl (2), TE containing 1 M ammonium sulfate (3), TE containing 50% ethylene glycol (4), TE containing 50% glycerol (5), TE containing 1 M NaCl and 50% ethylene glycol (6), TE containing 1 M ammonium sulfate and 50% ethylene glycol (7), TE containing 1 M NaCl and 50% glycerol (8), TE containing 1 M ammonium sulfate and 50% ethylene glycol (9), TE containing 1 M NaCl and 50% glycerol (10), TE containing 1 M ammonium sulfate and 50% ethylene glycol (11), TE containing 1 M NaCl and 50% glycerol (12), TE containing 1 M ammonium sulfate and 50% ethylene glycol (13), TE containing 1 M NaCl and 50% glycerol (14), and TE containing 1 M ammonium sulfate and 50% ethylene glycol (15). The secondary antibody and substrate were then reacted with the substrate. Fig. 3B. Control for the antibody stabilization assay. Conditions were equal to those in Fig. 3A except that the antigen-coated plate was treated with the stabilizing reagents before the BWG2 was reacted to establish that the stabilizing reagents were not removing the antigen from the plate.

Enzyme Stability Assays.

Effect of various concentrations of ammonium sulfate on enzyme activity. Control experiments demonstrated that the concentration of ammonium sulfate and ethylene glycol in the final reaction volume did not significantly affect the enzyme activity (data not shown).

Identification of Effective PEG/Salt Existing Concentrations by the ELISA-Eulsion Assay.

We used the ELISA-eulsion assay to examine varying concentrations of ethylene glycol and ammonium sulfate to determine the lowest concentration of each reagent that would not result in dispersion of the antigen-antibody complex (Fig. 3). When the ELISA-eulsion assay indicated that 50% ethylene glycol or 1 M ammonium sulfate could not be used, 10% ethylene glycol or 0.1 M ammonium sulfate was used. The effects of time and temperature on elution of RPNP II from BWG2-Sepharose were also examined. Purified wheat germ RPNP II was adsorbed to BWG2-Sepharose in a small batch assay. After thorough washing with TE containing 200 mM sodium sulfate, the BWG2-Sepharose was divided and treated with TE containing 0.2 M ammonium sulfate and 40% ethylene glycol at 5, 15, 25, and 30°C for 5, 10, 15, and 30 min. The Sepharose was quickly removed by centrifugation, and the supernatant fluid was diluted and assayed by the ELISA-eulsion method. As illustrated in Fig. 4, increasing the incubation time from 5 min to 1 h resulted in a decrease in recovery of enzyme. In addition, increasing the temperature from 5°C to 25°C did not result in an increase in recovery of enzyme at any temperature.

Identification of Existing Conditions by the Elisa-Eulsion Assay.

To help identify the conditions that maintain the antigen-antibody complex, we employed the ELISA-eulsion assay described in Materials and Methods. Because the colorimetric measurement of the primary antibody present after the color development is a decrease in optical density indicates that the enzyme-antibody complex has been disrupted, several reagents, or combinations of reagents, were tested to determine the antigen-antibody complex. The results in Fig. 3A indicated that possible eluting reagents were either 30% ethylene glycol or 50% glycerol (glycol), alone, numbers 1, 2, or a combination with salt numbers 6, 7, or 8. Numbers 1 and 6, in combination, eluted antigen-antibody complexes at 4°C, as determined by the ELISA-eulsion assay. However, all eluting reagents, like those used in prior experiments, did not result in increased recovery of enzyme. In addition, increasing the elution time from 15 min to 1 h did not result in an increase in recovery of enzyme at any temperature.
Fig. 5. ELISA-Ellution Assay Using Varying Concentrations of Ethylene Glycol and Ammonium Sulfate. Wheat germ RNA II that was immobilized on a protein A sepharose was reacted with RNAP16. The antigen-antibody complex was then challenged with ammonium sulfate (0 to 1.0 M) in the presence of 0, 20, 40, and 50% ethylene glycol for 20 min at room temperature.

Use of Peptide in the ELISA Ellution Assay
The synthetic peptide was also tested for the ability to disrupt the antigen-antibody complex. Peptide concentrations greater than 300 µg per mL were found to be effective in the ELISA ellution system (Fig. 8).

Fig. 8: ELISA-Ellution Assay Using the Synthetic Peptide. Wheat germ RNA II that was immobilized to polystyrene was reacted with RNAP16. The antigen-antibody reaction was then challenged with TE containing varying concentrations of synthetic peptide.
Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. Elution of active enzyme with protein stabilizing agents from a polyol-responsive monoclonal antibody.
N E Thompson, D B Aronson and R R Burgess


Access the most updated version of this article at http://www.jbc.org/content/265/12/7069

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/12/7069.full.html#ref-list-1