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A Method for the High Efficiency of Water-Soluble Carbodiimide-Mediated Amidation[1]

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Water-soluble carbodiimides are widely used for carboxyl–amine conjugation. However, extremely variable and low yields, obtained under a variety of conditions, have been a serious problem in the coupling. A simple method, optimizing various parameters of the coupling reaction, in which *N*-hydroxysuccinimide is included to assist the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride-catalyzed amidation reaction is described. A product yield of up to 90% is routinely achieved. © 1994 Academic Press, Inc.

Molecules with reactive amino or carboxyl groups may be coupled to proteins (1) or modified polymeric supports such as ECH- (2), AH-Sepharose 4B (3,4), or Affi-Gel 102 (5–7) under gentle conditions using water-soluble carbodiimides, e.g., 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC),[3] to form covalent conjugates via amide bonds. The reaction involves the intermediary formation of the activated *O*-acylurea derivative of the carbodiimide. A subsequent nucleophilic attack by the primary nitrogen of the amino compound brings about the formation of the amide linkage with the release of the soluble substituted urea (Scheme IA). The formation of *O*-acylurea occurs optimally at pH 4–5 (8); the intermediate has an extremely short half-life and rapidly undergoes hydrolysis or rearranges to give the *N*-acylurea adduct (9,10). The primary amino group of the nucleophile is predominantly protonated at this low pH and is rather unreactive. These limitations can severely restrict the yield of the product. Nevertheless, EDC has found much greater favor over the classical *N,N'*-dicyclohexylcarbodiimide in amide/peptide synthesis because of its high solubility in aqueous media and the easy removal of the excess reagent and the corresponding urea by washing the conjugated product with water and dilute acid.

The poor reactivity of the primary amino groups of the nucleophile at pH 4–5 and the instability of the acylurea intermediate in EDC-mediated conjugation of proteins are somewhat compensated in the preparation of solid affinity chromatographic media since proteins offer multivalent structures that result in an acceptable yield of the product with retention of functional characteristics for ligand–protein interaction. However, coupling efficiencies remain very low when monovalent compounds, especially those with limited solubilities in aqueous media, must be conjugated (11).

N-Hydroxysuccinimide (NHS) or its more polar derivative *N*-hydroxysulfosuccinimide (NHSS) reacts with carboxyl-containing compounds to give aminoacyl esters under facile conditions. The stable, active esters hydrolyze slowly in aqueous media compared with their rates of reaction with amino groups and can enhance the coupling efficiencies of carbodiimides for conjugating carboxylated compounds with primary amines (11–13; Scheme IB). The extremely low and variable yields of coupling *N*-carboxypentyldeoxynojirimycin (NCP-DNM), an inhibitor of glycoprotein-processing enzymes glucosidases I and II, to amino-substituted solid support Affi-Gel 102 via EDC-mediated conjugation (5–7) prompted us to optimize the conditions for this reaction. In this paper, we describe the combined use of NHS and EDC to link the monovalent butyric acid to

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[3] Abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; NHSS, *N*-hydroxysulfosuccinimide; Mes, 2-[N-morpholino]ethanesulfonic acid; DNM, 1-deoxynojirimycin; NCP-DNM, *N*-carboxypentyl-1-deoxynojirimycin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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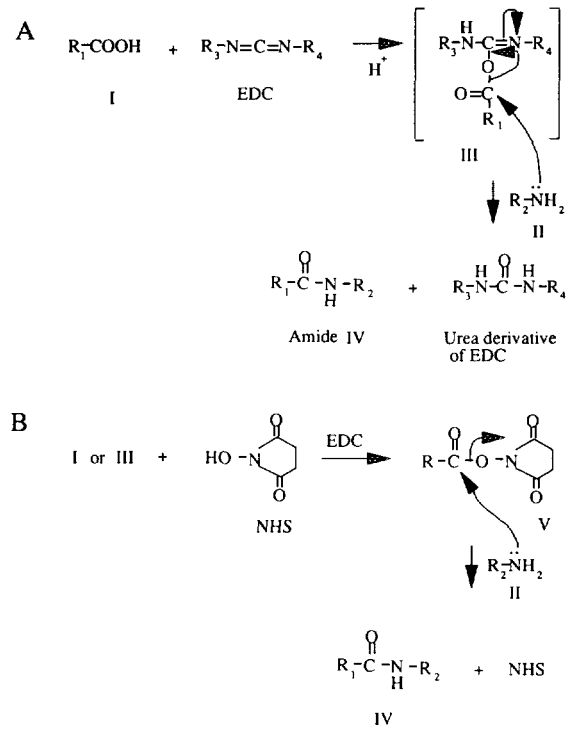

SCHEME I. EDC-mediated amidation reaction without (A) and with (B) the assistance of *N*-hydroxysuccinimide (NHS).

Affi-Gel 102, resulting in yields approaching 90%. The conditions developed were then used to conjugate NCP-DNM to Affi-Gel 102 with an identical yield. The affinity chromatographic matrix thus prepared was used to obtain pure glucosidase I from detergent-solubilized microsomes of the rat mammary gland.

MATERIALS AND METHODS

Reagents. EDC and Affi-Gel 102 were purchased from Bio-Rad Labs; NHS was obtained from Pierce Chemical Co. Butyric acid and 2-[N-morpholino]ethanesulfonic acid (Mes) were the products of Sigma. [^{14}C]Butyric acid (sp act, 55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO) and Na^{125}I (sp act, 17.4 Ci/mg) was from New England Nuclear Corp. Sephadex G-10 (PD-10) columns were from Pharmacia. 1-Deoxynojirimycin (DNM) was a kind gift of Drs. D. D. Schmidt and B. Garthoff, Bayer Chemical Works, Wuppertal, Germany; NCP-DNM was prepared as reported earlier (5). Polyclonal antibodies against rat mammary glucosidase I were a product of our laboratory. Alkaline phosphatase reagent kit was purchased from Vector Labs (Burlingame, CA). The 12-day lactating mammary tissue of the Sprague–Dawley rats was obtained from Hilltop Labs (Scottdale, PA).

Coupling of [^{14}C]butyric acid to Affi-Gel 102. Five micromoles of butyric acid and 1 μCi of [^{14}C]butyric acid were added to 2 ml of washed, preswollen gel matrix, equivalent to 25 μmol of reactive amino groups, suspended in 25 mM Mes, pH 6.5 (Buffer A), in a 15-ml screw cap tube. Coupling reactions were initiated by adding a solution of EDC and NHS to the same buffer to give a final reaction volume of 5 ml. The reaction tubes were rotated at room temperature for 6 h. The radioactivity in an aliquot of the uniformly suspended gel mixture was determined by scintillation counting while the remaining gel was washed extensively with 100 mM phosphate buffer, pH 6.8, over a sintered glass funnel. Next, the gel was washed with the same buffer containing 1% Triton X-100 until the radioactivity in the eluate reached background levels. The washed gel was suspended in 5 ml of Buffer A and its radioactivity determined in an aliquot to calculate the [^{14}C]butyric acid conjugated to the gel.

The coupling conditions were optimized with respect to pH, ratio of NHS to EDC and carboxyl to amino groups, time, and temperature (room temperature and 4°C).

Coupling of N-carboxypentyl-1-deoxynojirimycin (NCP-DNM)to Affi-Gel 102. Ten milligrams of NCP-DNM (35 μmol) was conjugated to 28 ml (350 μmol of NH_2 groups) of Affi-Gel 102 in a reaction mixture containing 1.56 mmol of NHS and 23.4 mmol of EDC (NHS/EDC ratio of 1:15) in 25 mM Mes, pH 6.5, in a final volume of 70 ml. The tube was rotated for 24 h at room temperature. The gel was washed with 100 mM phosphate buffer, pH 6.8, and equilibrated with the same buffer containing 0.8% Lubrol.

Affinity purification of glucosidase I. This was carried out as described previously (6,7). Briefly, a 0.8% Lubrol-solubilized preparation of the microsomal proteins of the rat mammary gland was adsorbed to the DNM affinity matrix described above and eluted with 10 mM DNM in 100 mM phosphate buffer, pH 6.8, containing 0.8% Lubrol. The DNM was removed from the eluted enzyme by gel filtration on a PD-10 column. An aliquot of the enzyme was checked for activity; another aliquot was subjected to 10% SDS–PAGE (14) followed by silver staining of the gel.

Other methods. Western blots of purified glucosidase I were probed with anti-glucosidase I antibody as before (7). Protein was determined by the bicinchoninic acid (BCA Kit, Pierce Chemical Co.) with BSA as standard.

RESULTS

Several different parameters and conditions were examined in which proportions of NHS and EDC were

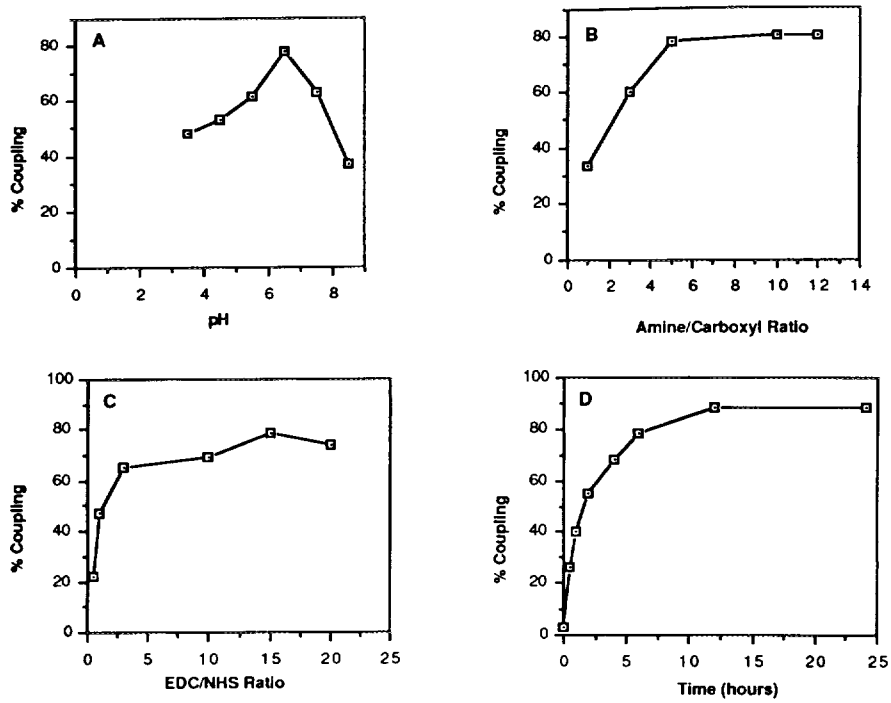

FIG. 1. Effects of different variables, viz., pH (A), amine/carboxyl ratio of Affi-Gel 102 to [^{14}C]butyric acid (B), EDC/NHS ratio (C), and time of coupling at room temperature for an amine/carboxyl ratio of 10:1 and EDC/NHS ratio of 15:1 (D), on the coupling reaction. Other details are given in the text.

varied to obtain the maximum yield for the conjugation of [^{14}C]butyric acid to Affi-Gel 102 at room temperature. The optimum pH for coupling at room temperature for 6 h was found to be 6.5 (~80% yield, Fig. 1A). When the carboxyl to amine ratio was varied, there was a linear increase in conjugation up to a ratio of 1:5; a further increase in this ratio up to 1:12 resulted in only a slight increase in product formation (Fig. 1B). When the concentration of EDC was varied keeping the carboxyl to amine ratio of 1:10, the maximum coupling (~80%) was achieved at an NHS/EDC ratio of 1:15; beyond this, there was a slight drop in the yield of the product (Fig. 1C). With respect to time, the initial linear rate gradually leveled off, giving a maximum yield of ~90% after 12 h of reaction (Fig. 1D). On the basis of these data, the optimum conditions for the conjugation of carboxyl group of butyric acid to the amino function of Affi-Gel 102 at ratio 1:10 appeared to be pH 6.5 and an NHS/EDC ratio of 1:15 for 12 h or longer.

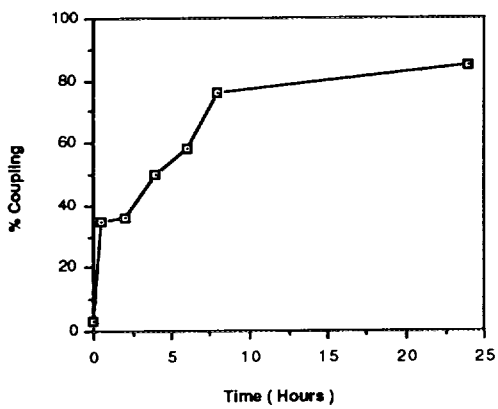

FIG. 2. Conjugation of [^{14}C]butyric acid to Affi-Gel 102 at 4°C under optimum conditions, as given under Results.

For labile biological compounds, especially proteins, it is desirable to perform the conjugation reaction at 4°C to minimize the loss of functional characteristics. When the optimal parameters defined above were applied in the reaction conducted at 4°C, a coupling yield of 85% after 24 h was obtained (Fig. 2).

Next, the coupling of NCP-DNM to Affi-Gel 102 was carried out at 4°C under the above standardized conditions. A detergent-solubilized extract of rat mammary gland microsomal proteins was adsorbed and eluted from this affinity matrix as described under Materials and Methods. After removal of DNM on a PD-10 column, the eluted fraction was tested (6) and found to have glucosidase I activity (not shown). The eluted fraction comprised a single polypeptide of 85 kDa, as exam-

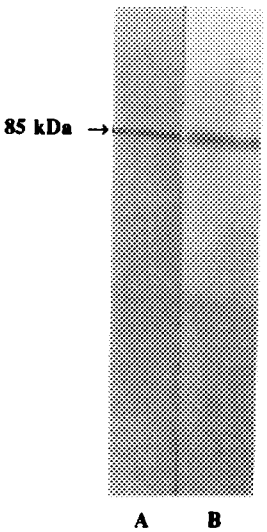

FIG. 3. SDS–PAGE (Lane 1) and Western blot (Lane 2) of rat mammary glucosidase I purified on *N*-carboxypentyl-1-deoxynojirimycin Affi-Gel 102 column.

ined by SDS–PAGE and silver staining of the gel. When transferred to nitrocellulose, the 85-kDa polypeptide was recognized by a monospecific polyclonal anti-glucosidase I antiserum (Fig. 3), thus confirming the integrity of the affinity chromatographic matrix.

DISCUSSION

Our laboratory had been using an affinity chromatography matrix, 1-deoxynojirimycin-*N*-carboxypentyl–Affi-Gel 102, for the rapid purification of the glycoprotein-processing enzyme glucosidase I (6,7). The matrix was prepared by EDC-facilitated amidation of NCP-DNM to Affi-Gel 102, yielding about 10% conjugation with [^{14}C]butyric acid by the method of Hettkamp *et al.* (4). Recent attempts to remake the matrix for further studies on the enzyme resulted in extremely low yields. DNM, like many monovalent ligands, has no chromophore to directly monitor the coupling reaction. Also, a radioactive form of the antibiotic that might be used to determine the product yield by tracer estimation is not available. The exorbitant cost of the commercially available reagent led us to undertake a systematic evaluation of the amidation reaction to optimize the coupling protocol to obtain the affinity matrix.

A search of the literature revealed that even though the water-soluble EDC had been widely used for carboxyl–amine conjugation, a variety of buffers at different concentrations and at pH 4–5 were employed in the reported studies. It has been shown that the phosphate buffer commonly used in such conjugations is incompatible with the stability of the acylurea intermediate of EDC (reviewed in Ref. 13). Also buffers such as acetate and citrate cannot be used because these would make mixed anhydrides with the carboxyl reactant and drive the nucleophilic amino reagent into side reactions and by-products. The different concentrations of EDC and the conditions of the coupling reaction reported in the literature have resulted in highly variable yields for linking haptens to carriers in the preparation of immunizing antigens, protein–ligand conjugates, and affinity chromatographic supports.

In a preliminary report, Staros *et al.* (11) improvised on the EDC-mediated amidation by including NHSS in the reaction milieu. In addition to a direct acylation, such a mixture of the two reagents would also allow for transacylation from the *O*-acylurea to NHSS to give the active acyl-NHSS ester. The latter, being more stable than the EDC-derivative, would enhance the efficiency of coupling. A conjugation of the monovalent [^{14}C]-glycine to keyhole limpet hemocyanin gave a product yield of ~21% at 5 mM NHSS versus 1.4% in its absence; further increase in the level of NHSS caused a steady decline in amide formation. Using a similar approach, more recently Moffett *et al.* (13) conjugated radioactively labeled neuronal molecules *N*-acetylaspartate and *N*-acetylaspartylglutamate to BSA for the preparation of immunizing antigens. These workers optimized the conditions for the coupling reaction in the presence of NHS; however, product yields conspicuously remained low, i.e., less than 20%.

While NHSS-derivatized aminoacyl esters have greater solubility in aqueous medium than the corresponding NHS-derivatized esters, NHS gave us a greater yield of the product. In this study, we have defined the optimal conditions for the EDC-mediated amide formation in the presence of NHS and shown that product yields approaching 90% can be obtained. The amidation proceeds equally well at 4°C, a temperature that is more suitable than room temperature (22–23°C) for coupling proteins with retention of functional activity. A successful application of the method to purification of glucosidase I by affinity chromatography on a column of 1-deoxynojirimycin-*N*-carboxypentyl–Affi-Gel 102 is also presented. It is expected that the protocol will find widespread use in the preparation of amides for biological research.

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