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TANDEM USE OF CARBOXYPEPTIDASE Y REACTOR AND DISPLACEMENT CHROMATOGRAPH FOR PEPTIDE SYNTHESIS

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SUMMARY

A packed-bed enzyme reactor with immobilized carboxypeptidase Y was used in tandem with a displacement chromatograph for the preparation of N-benzoyl-L-arginyl-L-methioninamide, from N-benzoyl-L-arginine and L-methioninamide. The pumps and valves of the coupled enzyme reactor and displacement chromatograph were controlled by a microprocessor. The enzyme was immobilized on microparticulate amino-silica by glutaraldehyde and packed into a 60 × 4.6 mm I.D. column. The packed-bed reactor was used in the recirculating mode and components of the reaction mixture were subsequently separated by displacement chromatography on a 250 × 4.6 mm octadecyl-silica column using butoxyethoxyethanol as the displacer. Unreacted L-methioninamide was returned to the reaction mixture. Both the progress of the reaction and the extent of separation by displacement chromatography were monitored by high-performance liquid chromatographic analysis. The system was designed so that enzymatic peptide synthesis, separation by displacement chromatography, and column regeneration were carried out simultaneously by using two identical columns in parallel. An amount of 460 mg of N-benzoyl-L-arginyl-L-methioninamide having purity greater than 99% could be obtained in 24 h with this system. The tandem operation of the enzyme reactor and liquid chromatograph operated in the displacement mode offers a means for the synthesis and purification of peptides.

INTRODUCTION

Despite advances in chemical[1,2] and solid phase peptide synthesis[3,4], there is a growing interest in enzymatic peptide synthesis for the preparation of biologically active peptides[5–7] due to the stereospecificity of the enzymatic peptide bond formation.

The serine exopeptidase, carboxypeptidase Y (CPY) from baker's yeast, has been shown to be a general catalyst for peptide synthesis[8–13] and the use of this enzyme in immobilized form (Imm-CPY) has recently been investigated in our lab-

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oratory[14]. In the course of this work we have found that displacement chromatography[15–17] offers significant advantages over elution chromatography in separating the component of the relatively dilute peptide synthesis mixtures[18]. The present work describes the tandem synthesis of N-benzoyl-L-arginyl-L-methioninamide (Bz-Arg-Met-NH_2) by Imm-CPY and product isolation by displacement chromatography.

EXPERIMENTAL

Materials

Carboxypeptidase Y, specific activity of 135 units/mg, was a gift from Carlsberg Biotechnology (Copenhagen, Denmark). Octadecylsilica (10 μm) (lot No. 3223-45A), a gift from Amicon (Danvers, MA, U.S.A.), was packed into 250 × 4.6 mm columns. N-Benzoyl-L-arginine (Bz-Arg) and N-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) were obtained from Sigma (St. Louis, MO, U.S.A.). Butoxyethoxyethanol was purchased from Fisher (Pittsburgh, PA, U.S.A.). The support used for enzyme immobilization was 10-μm Vydac silica having a specific surface area of 100 m^2/g and mean pore diameter of 330 Å (Separation Group, Hesperia, CA, U.S.A.). Z-6050 polyamino-functional silanizing agent was obtained from Dow (Midland, MI, U.S.A.). Glutaraldehyde, 50% (w/w) aqueous solution, was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Sodium borate, methanol, and EDTA were supplied by Fisher. Distilled water was prepared with a Barnstead distilling unit.

Apparatus

Fig. 1 shows a schematic of the tandem reactor–chromatograph system. V_1, V_2, and V_5 are Model 7030 three-way valves (Rheodyne, Cotati, CA, U.S.A.) and V_3 and V_4 are Model 7040 four-way valves (Rheodyne). The operation of these valves is described in Table I. Two recirculating water-baths, Lauda Model K-2/R (Brinkmann, Westbury, NY, U.S.A.) were used independently to control the temper-

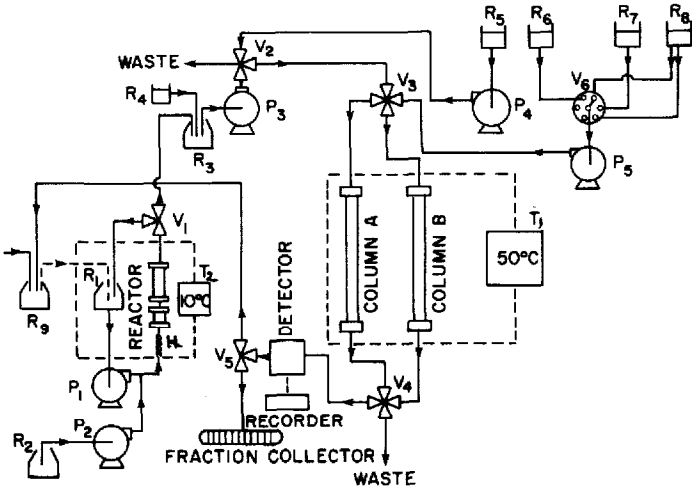

Fig. 1. Flow sheet of the tandem enzyme reactor–displacement chromatograph. The symbols are R, reservoir; P, pump; V, valve; T, thermostated baths; H, heat exchanger; E, enzyme reactor; and G, guard column. See further details in text.

TABLE I

FLOW CONTROL BY VALVE SWITCHING

Valve	*Position*	
	I	*II*
V_1	Reactor effluent to recycle reservoir R_1	Reactor effluent to pH adjustment reservoir R_3
V_2	Displacer to waste: feed to V_3	Feed to waste; Displacer to V_3
V_3	Regenerants to column A; displacer or feed to column B	Displacer or feed to column A; regenerants to column B
V_4	Effluent of column A to detector; effluent of column B to waste	Effluent of column A to waste; effluent of column B to detector
V_5	Effluent from detector to fraction collector	Effluent from detector to Met-NH_2 collection reservoir R_9

ature of the enzyme reactor and the two identical 250 × 4.6 mm columns packed with 10-μm octadecylsilica (Amicon). The temperature of the column undergoing displacement was maintained at 50°C by directing the recirculating water-bath flow into a water-jacket surrounding the column, while circumventing the column undergoing regeneration. The flow through the column water-jackets was manually controlled. The reactor, E, and the reservoir, R_1, were kept at 10°C.

The immobilized enzyme was packed into a 60 × 4.6 mm column and held by two 2-μm stainless-steel fritted disks to obtain the enzyme reactor, E. A 10 × 4.6 mm guard column packed with 5-μm Partisil silica (Whatman), a 200-cm heat exchanger coil of 0.25 mm I.D. and 1.59 mm O.D. No. 316 stainless-steel capillary tubing were placed before the inlet of the reactor as shown in Fig. 1. Reservoir R_1, containing the reaction mixture, was stirred with a Model PC magnetic stirrer (Corning Glass, Corning, NY, U.S.A.).

A Model A-30-S pump (Eldex, Menlo Park, CA, U.S.A.), P_3, was used for the introduction of the 12-ml feed from reservoir R_3. Reservoir R_4 contained 5 ml of 85% phosphoric acid which was used to adjust the pH of the feed as described below. One Model 110A pump (Altex-Beckman, San Ramon, CA, U.S.A.), P_1, was used to recirculate the 15-ml reaction mixture contained in reservoir R_1 and another Model 110A pump, P_4, generated the flow of the displacer solution from the 1-l reservoir R_5. A Model B-100-S pump (Eldex), P_2, was used to pump water from the 100-ml reservoir R_2 through the reactor at the end of the synthesis reaction. A Model 1016/AA-94 dual piston pump (Eldex), P_5, was used to pump water, methanol, and 0.1 *M* phosphoric acid sequentially from the respective reservoirs R_6, R_7 and R_8, each of 1-l volume, through the column during the regeneration step by the use of

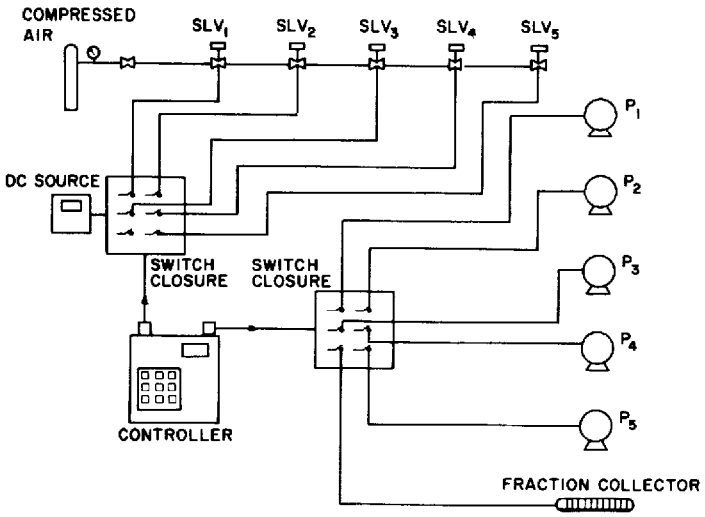

Fig. 2. Schematic of microprocessor control of the valves and pumps employed in the tandem enzyme reactor–displacement chromatograph system. SLV represents pneumatically activated solenoid valve.

a Model II 6-way selector valve (Eldex), V_6. A TriDet detector (Perkin-Elmer, Norwalk, CT, U.S.A.) monitored the column effluent and the signal was recorded with a Model R-100 A strip chart recorder (Perkin-Elmer). An Ultrarac fraction collector (LKB, Bromma, Sweden) was used to collect 150-μl fractions from the column effluent except during the elution of Met-NH_2 which was directed to the 100-ml reservoir R_9 by valve V_5.

The sequencing of all switching valve operations via a Model 70-01 pneumatic actuator (Rheodyne) and the delivery of power to the pumps, detector and fraction collector was controlled by an Eldex Chromat-a-trol as shown in Fig. 2. A Model 7163 solenoid valve kit (Rheodyne) with a switch closure module (Eldex) controlled the operation of the valves as shown in Table I. A power module (Eldex) along with two home made 12-V power supplies were used for the operation of all the equipment.

Procedures

Synthesis of Bz-Arg-Met-NH_2. The preparation of the amino-silica support from 10-μm Vydac wide pore (330 Å) silica gel with Z-6050 polyamino-functional silane, the activation of the support by glutaraldehyde and the immobilization of CPY were carried out as described elsewhere[14]. A 60 × 4.6 mm packed-bed reactor with Imm-CPY was employed for the synthesis of Bz-Arg-Met-NH_2. The 15-ml reaction mixture in reservoir R_1 contained 10 m*M* Bz-Arg-OEt and 50 m*M* Met-NH_2 in 50 m*M* borate buffer, pH 9.0, with 5 m*M* EDTA. It was recirculated through the reactor by pump P_1 at a flow-rate of 4 ml/min and a temperature of 10°C. After 2 h, valve V_1 was set to position II directing the flow of the reaction mixture into reservoir R_3 for pH adjustment. After 12 ml of the reaction mixture accumulated in reservoir R_3, pump P_1 was turned off, leaving 3 ml of the reaction mixture in reservoir R_1 so that pump P_1 remained primed for the subsequent reaction. Pump P_2 was then turned on and water was pumped through the reactor at a flow-rate of 1 ml/min to

transfer the remaining reaction mixture from the reactor into reservoir R_3. After 1 minute the reactor effluent was directed to waste and the reactor was washed with water for an additional 4 min. A volume of 150 μl of 85% phosphoric acid from reservoir R_4 was then added to the content of reservoir R_3 in order to bring the pH to 2.5. A complete reaction cycle took 2 h and 15 min.

Subsequent cycles were initiated by first placing 15 ml of fresh reaction mixture into reservoir R_1, then turning on recirculating pump P_1 to pump the mixture through the reactor. After the first 1 ml of the reactor effluent was directed to waste to eliminate the water present in the reactor, valve V_1 was set to position I to initiate the recirculation of the reaction mixture.

Displacement chromatography. Displacement chromatography was alternately carried out with columns A and B using the system shown in Fig. 1. While one column was separating the other was being regenerated. The same procedure was used for displacement chromatography with both columns. The column was first equilibrated with a 0.1 *M* phosphoric acid solution in water, pH 2.2, used as the carrier. Valve V_2 was then set to position I and the reaction mixture was pumped from reservoir R_3 into the column by pump P_3 at a flow-rate of 0.5 ml/min at 25°C. The feed was directed into column A or B by switching valve V_3 to position II and I, respectively. The effluent of the column was directed to either the detector or to waste by valve V_4 as described in Table I. After 11.5 min of introduction of the feed, the front of Met-NH_2 appeared in the detector and valve V_5 was switched to direct the flow from the detector effluent to reservoir R_9.

After 26 min of introduction of the feed into the column, pump P_3 was turned off and the column temperature was raised to 50°C as described above. The displacer was then introduced into the column at a flow-rate of 0.1 ml/min by pump P_4 upon setting valve V_2 to position II. After 45 min of introduction of the displacer, the Met-NH_2 had eluted from the column and valve V_5 was set to position I directing the flow from the detector effluent to the fraction collector. Following the introduction of the displacer for an additional 40 min, Bz-Arg appeared in the effluent and the collection of 150-μl fractions began. The fractions containing Bz-Arg, Bz-Arg-OEt, and Bz-Arg-Met-NH_2 were collected over the next 25 min until the displacer front emerged from the column and pump P_4 was turned off. The entire separation process took 2 h and 15 min.

Regeneration of the chromatographic column. During the separation process described above, the other column was regenerated by pumping various regenerants in succession at a flow-rate of 2 ml/min using pump P_5 with valves V_3 and V_4 in the appropriate positions given in Table I to direct the regenerating column effluent to waste. Valve V_6 was used to select a given regenerant. The regeneration protocol for removing the displacer from the column called for sequential perfusion with 30 ml of water, 150 ml of methanol, 46 ml of water, and 46 ml of carrier in 2 h and 16 min. During the regeneration process the flow from the recirculating water bath was not directed to the regenerating column and the column was cooled to room temperature by the flow of regenerants.

High-performance liquid chromatographic analysis. For analytical work a Model LC 250/1 pump (Kratos, Westwood, NJ, U.S.A.), a Model 7010 sampling valve with a 20-μl sample loop (Rheodyne) a Model SF 770 UV detector (Kratos) and a Model CI-10 integrator (LDC, Milton-Roy, Riviera Beach, FL, U.S.A.) were

used with a 5-μm C_8 250 × 4.6 mm column (IBM, Danbury, CT, U.S.A.). A 50 m*M* phosphate buffer, pH 3.0, containing 40% (v/v) methanol was used as the eluent at 25°C. The reaction progress at 15-min intervals and the composition of the fractions obtained by displacement development were measured. Samples of 5 μl were taken, diluted with the eluent and 20-μl aliquots were injected. The column effluent was monitored at 254 nm where the Bz protecting group has a strong absorbance. Peak areas were used in quantitative analysis.

RESULTS AND DISCUSSION

The coupling of a packed-bed Imm-CPY recycle reactor[14] and a displacement chromatograph[18] presented here is an extension of the work of El Rassi and Horváth[19]. In that work, a tandem immobilized ribonuclease T_1 reactor–liquid chromatograph system was employed for the preparation of nucleic acid fragments. The present work adapts the microprocessor controlled unit for enzymatic peptide synthesis and employs two chromatographic columns in parallel to enable simultaneous separation and regeneration via column switching.

The flow of reagents throughout the system was directed using a matrix of pneumatically actuated switching valves controlled by the microprocessor. In order to synchronize the individual steps and thus facilitate the control of the overall process, conditions for the peptide synthesis reaction, displacement chromatographic purification, and column regeneration were selected so that their times were approximately the same. This was accomplished using two identical separation columns in parallel, with one undergoing regeneration and the other separating the components of the reaction mixture by displacement chromatography. While the timing of column regeneration was relatively flexible, the timing of displacement purification and enzymatic peptide synthesis were less so as described below. The reaction volume was chosen so that the entire reaction mixture could be used as the feed for a single displacement chromatographic run. While the work presented here is on the preparation of Bz-Arg-Met-NH_2, this system could be used for alternative peptide syntheses[14] by changing the operational parameters of the system.

Bz-Arg-Met-NH_2 synthesis

The CPY catalyzed synthesis of Bz-Arg-Met-NH_2 from Bz-Arg-OEt and Met-NH_2 was selected for this study since both the kinetics and stability of the immobilized enzyme for this reaction have been investigated in conjunction with the enzymatic synthesis of L-methionyl-L-leucyl-L-phenylalanine[14]. Operating conditions were established in that work which retained the enzymatic activity of the reactor after several syntheses[14].

As stated above, the criterion for selecting the volume of reaction mixture was that the volume of a fixed concentration of reactants would match the column capacity in displacement chromatography. Previous experiments had indicated that 15 ml of this reaction mixture could be separated by displacement chromatography with one 25-cm C_{18} column in approximately 2 h using butoxyethoxyethanol as the displacer[18]. Accordingly, a 15-ml reaction volume was selected for this study.

Fig. 3 shows the progress of the reaction in the present system which resulted in a 65% conversion of substrate with 80% selectivity for Bz-Arg-Met-NH_2 in 2 h.

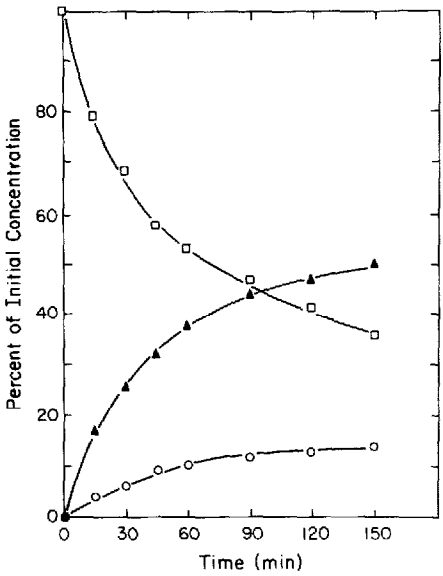

Fig. 3. Time course of Bz-Arg-Met-NH_2 synthesis by the recirculating enzyme reactor. Reaction mixture, 15 ml of 10 m*M* Bz-Arg-OEt and 50 m*M* Met-NH_2 in 50 m*M* borate buffer, pH 9.0, with 5 m*M* EDTA. Reactor, 60 × 4.6 mm packed-bed reactor with Imm-CPY; flow-rate, 4 ml/min; temperature, 10°C. □, Bz-Arg-OEt; ▲, Bz-Arg-Met-NH_2; ○, Bz-Arg.

Product inhibition[14] caused a decrease in the rate of product formation after 2 h. The reaction time was set to 2 h and 10 min to match the time of the displacement run. A water wash of 5 min was added to remove any precipitate that may have formed in the reactor. The peptide synthesis reaction was found to be very reproducible with respect to the yield of product in the several repetitions conducted.

Since the substrate was not completely converted, the unreacted components were also separated by displacement chromatography so that they could be reused in subsequent synthesis reactions. The recycling of the excess Met-NH_2 was controlled in this system. However, unreacted Bz-Arg-OEt could also be recycled by using a similiar set of commands.

Displacement chromatography

The conditions for displacement chromatography of the reaction mixture have been established previously[18] and Fig. 4 shows the separation of the components obtained with the present tandem system. Most of Met-NH_2 eluted during the introduction of the feed and the remainder of the Met-NH_2 eluted within 45 min after the introduction of the displacer. After 85 min of the introduction of the displacer, Bz-Arg emerged as the first band of the displacement train followed by the bands of Bz-Arg-OEt and Bz-Arg-Met-NH_2. The zones were well separated and the displaced components were concentrated during the separation process from feed concentrations of 1.4, 3.6 and 5 m*M* to concentrations of 55, 73, and 104 m*M* of Bz-Arg, Bz-Arg-OEt and Bz-Arg-Met-NH_2, respectively. The recoveries of Met-NH_2, Bz-Arg, Bz-Arg-OEt and Bz-Arg-Met-NH_2, at greater than 99% purity, were 100, 87, 86 and 93%, respectively.

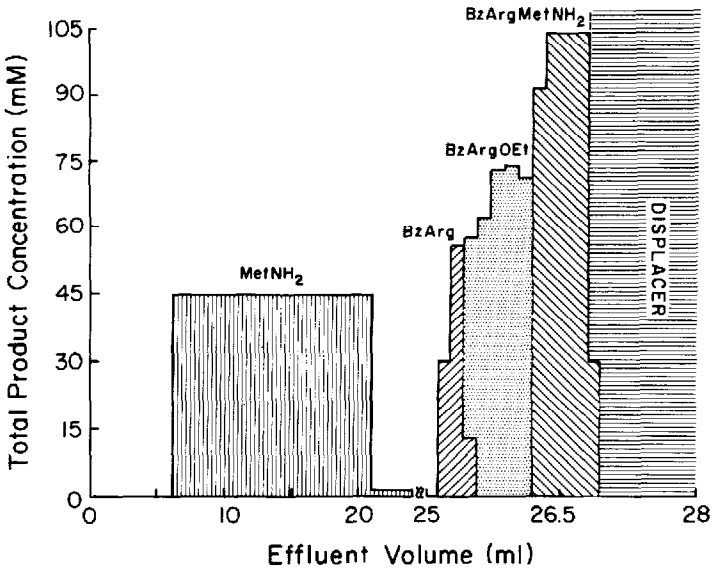

Fig. 4. Displacement chromatogram of the reaction mixture at the end of Bz-Arg-Met-NH_2 synthesis. Column, 10-μm Amicon ODS (250 × 4.6 mm); carrier, 0.1 *M* phosphate buffer, pH 2.2; displacer, 40 g/l butoxyethoxyethanol in the carrier; temperature, 50°C; flow-rate, 0.5 ml/min and 0.1 ml/min for the feed and displacer, respectively. Feed, 13 ml of 5 m*M* Bz-Arg-Met-NH_2, 3.6 m*M* Bz-Arg-OEt, 1.4 m*M* Bz-Arg and 45 m*M* Met-NH_2. Volume of fractions, 150 μl.

In our system, which is based solely on control of the timing of events, the reproducibility of the events is an important criterion. With the two chromatographic columns displacements were very reproducible. The Bz-Arg zone emerged at 85 min ± 10 s when the separation process was repeated six times. In one cycle, the system produced 45 mg of pure Bz-Arg-Met-NH_2 so that 460 mg of pure Bz-Arg-Met-NH_2 was obtained in a 24-h period.

Microprocessor operation files

The tandem reactor–displacement chromatograph system was controlled by the Eldex Chromat-a-trol as described in the Experimental section and the following processes were regulated: synthesis of Bz-Arg-Met-NH_2, introduction of the reaction mixture into reservoir R_3 for pH adjustment, pumping of the feed into the displacement column, separation of the components by displacement chromatography, collection of the purified excess Met-NH_2 in reservoir R_9, collection of the purified reaction products using a fraction collector, and regeneration of the displacement column. Three files were written to control various stages of the simultaneous enzymic reaction, displacement development, and column regeneration. Start-up of the tandem system consisted of a simultaneous synthesis reaction and regeneration of column A using the file shown in Table II to control the timing of each event in these operations. The orientations of the switching valves are listed in Table I and the positions of the regenerant selection valve V_6 for water, methanol, water, and carrier are denoted by $V_{6\text{-}1}$, $V_{6\text{-}2}$, $V_{6\text{-}3}$ and $V_{6\text{-}4}$ respectively.

After the start-up cycle given in file 1, the system was subsequently operated

TABLE II

CONTROLLER FILE 1: PEPTIDE SYNTHESIS AND REGENERATION OF COLUMN A

Time			*Event*	
h	*min*	*s*	*Position*	*Valve/pump*
00	00	00	II	V_4
00	00	00	I	V_3
00	00	00	I	V_6
00	00	00	On	P_5
00	00	00	II	V_1
00	00	00	On	P_1
00	00	15	I	V_1
00	15	00	II	V_6
01	30	00	III	V_6
01	53	00	IV	V_6
02	07	00	II	V_1
02	10	00	Off	P_1
02	10	20	On	P_2
02	15	00	Off	P_2
02	16	00	Off	P_5

according to file 2 which was written to control two subsequent cycles. In the first cycle, peptide synthesis, displacement chromatography using column A and regeneration of column B occurred simultaneously, whereas in the second cycle the role of the two chromatographic columns was reversed. In the course of this work, the two cycles of file 2 were repeated 3 times to test the reproducibility of the system.

The last two cycles of the coupled system were controlled by a third file which was the same as file 2 except without reaction and use of column B during the second cycle. The separation of the reaction mixture from the last synthesis of file 2 and the regeneration of both chromatographic columns were carried out according to the third file.

CONCLUSIONS

The synthesis of Bz-Arg-Met-NH_2 was carried out by an immobilized carboxypeptidase Y reactor coupled to a displacement chromatograph. Two identical chromatographic columns were used in parallel, each alternating between separation and regeneration steps. The system was designed so that the synthesis, separation, and regeneration operations took place simultaneously.

While the tandem reactor–chromatograph was not fully automated, the automation of the remaining manual steps would be relatively straightforward. Since control of the system was based solely on the timing of the events, appropiate feedback controls would be needed to facilitate smooth operation over an extended period of time. Whereas the work presented here demonstrates that the tandem system has found use in the preparation of pure peptides it can also serve as a model for similar instruments suitable for the *ad hoc* preparation or continual supply of complex biochemical substances in the laboratory.

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