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PRODUCTION OF "INTERNAL SURFACE REVERESED-PHASE" SUPPORTS: THE HYDROLYSIS OF SELECTED SUBSTRATES FROM SILICA USING CHYMOTRYPSIN

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SUMMARY

The degree of hydrolysis of substrates attached to silica supports with α-chymotrypsin has been evaluated relative to the production of "internal surface reversed-phase" supports. The peptide substrates N-*tert.*-butoxycarbonyl-L-phenylalanine (Boc-L-Phe), N-carbobenzoxy-L-valine-L-phenylalanine, N-acetyl-L-phenylalanine (acetyl-L-Phe) and N-benzoyl-L-phenylalanine as well as phenylpropionic acid were attached to glycerylpropyl-bonded silica via a diamine spacer using 1,1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a coupling catalyst. The products released from the silica support on enzyme treatment were quantified by high-performance liquid chromatography. Boc-L-Phe and acetyl-L-Phe were successfully cleaved from the rigid silica matrix in high yields, whereas the remaining substrates were hydrolyzed to a lesser extent.

INTRODUCTION

The separation and quantification of drugs in serum and plasma by conventional high-performance liquid chromatography (HPLC) generally requires the removal of proteins prior to sample injection onto small particulate columns. Although the sample clean-up procedure has the advantage of removing endogenous species and preconcentration the drugs, the procedures are time consuming and difficult to automate.

Recently, the introduction of a new concept in HPLC packing, referred to as internal surface reversed-phase (ISRP) supports, enables the direct injection of serum or plasma samples, thus eliminating the need of sample preparation[1–3]. The ISRP supports consist of a hydrophobic partitioning phase bound only to the internal surface of porous silica particulates (5 μm diameter), while the external surface is rendered non-adsorptive to proteins via a glycerylpropyl-bonded phase. Since the mean pore diameter of the ISRP supports is small (52 Å), plasma proteins are excluded from the internal region of the porous silica[4]. The hydrophobic partitioning phase moieties are removed from the external surface by enzyme cleavage. The drugs being small penetrate the ISRP supports, interact with the partitioning phase, and undergo separation.

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The first ISRP supports were prepared by derivatization of porous silica with glycerylpropyl silane followed by the attachment of glycine-L-phenylalanine-L-phenylalanine to a finite fraction of the glycerylpropyl groups[1,2]. The phenylalanine groups were removed from the external surface by enzyme cleavage with carboxypeptidase A. A second type of ISRP supports has been prepared by first derivatizing silica with an amine spacer to which hydrophobic partitioning molecules are subsequently attached. The hydrophobic groups on the external surface are then removed with chymotrypsin[3]. The external surface is then rendered non-adsorptive to proteins by attaching glycol groups to the free amine spacers. The advantage of the latter method, for the production of ISRP supports, is a greater partitioning phase coverage.

The following work evaluates the ability of chymotrypsin to cleave various partitioning substrates from the external surface of silica supports. This knowledge is valuable to the development of new ISRP supports with varying partitioning phase selectivities.

EXPERIMENTAL

HPLC apparatus

The molecular substrates released on hydrolysis were separated and quantified with an HPLC system consisting of an Altex Ultrasphere IP reversed-phase ODS column (25 cm × 4.6 mm I.D.) through which a mobile phase of methanol–0.1 *M* orthophosphate, pH 2.9 (10:90) was pumped with an LDC Model 396 single-piston pump. The sample injector consisted of a Reodyne 7010 valve equipped with a 20-μl loop. The outlet of the column was connected to a Beckman Model 153 fixed-wavelength detector with a 254-nm filter and an 8-μl flow cell.

Reagents

N-*tert.*-Butoxycarbonyl-L-phenylalanine (Boc-L-Phe) was purchased from Chemical Dynamics. N-Carbobenzoxy-L-valine-L-phenylalanine (Cbz-L-Val-L-Phe), N-acetyl-L-phenylalanine (acetyl-L-Phe), N-benzoyl-L-phenylalanine (Bz-L-Phe) and 1,1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma. The 1,4-diaminobutane was purchased from Aldrich. The controlled-pore glass Glycophase CPG40 (37–74 μm particle diameter, 40 Å nominal pore size) was obtained from Pierce. Type II bovine pancreas α-chymotrypsin (salt free and three times crystallized) was purchased from Sigma. The chymotrypsin activity was periodically measured by standard procedures using benzoyl-L-tyrosine ethyl ester as a standard substrate[5]. The enzyme always showed more than 90% of the activity indicated by the supplier.

Silica support derivatization

Four different peptide substrates and one synthetic molecule (illustrated in Fig. 1) were bonded to a silica support via a 1-(N-diaminobutyl)-2-oxypropyl ethane spacer (Fig. 2). The coupling procedure (Fig. 2), described here with Boc-L-Phe as the substrate, was identical for all substrates. The starting support silica (Glycophase CPG40) consisted of controlled-pore glass to which a hydrophillic glycerylpropyl phase had been attached. In the first step of the synthesis, the glycerylpropyl phase

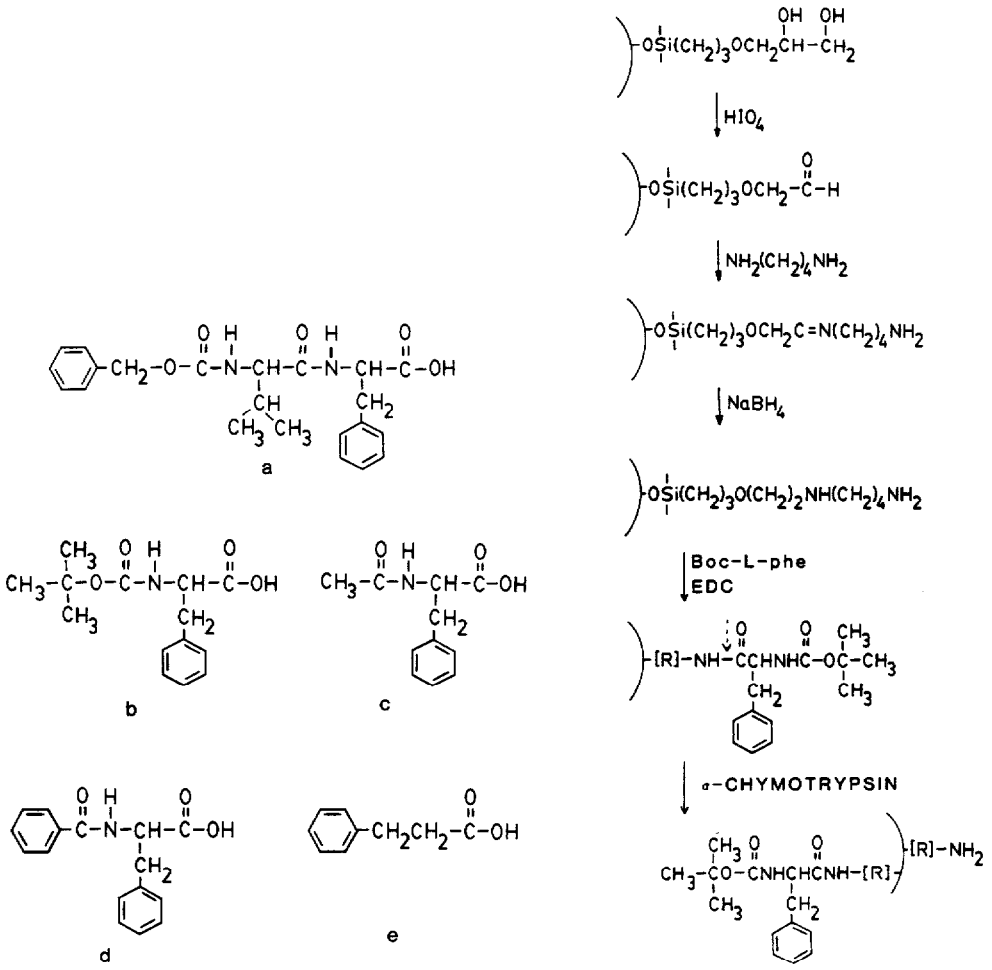


Fig. 1. Hydrophobic substrates. a = Cbz-L-Val-L-Phe; b = Boc-L-Phe; c = acetyl-L-Phe; d = Bz-L-Phe; e = 3-phenylpropionic acid.

Fig. 2. Synthesis scheme illustrating attachment of amine spacer to glycerylpropyl-bonded phase, bonding of substrate to amine spacer, and removal of substrate from external silica surface with chymotrypsin.

moieties were oxidized to an aldehyde. Typically, 1.5 g of the glycol support was placed in a dark flask and covered with 125 ml of oxidizing agent[6]. The suspension containing the silica matrix was shaken for 2 h, then thoroughly washed with water.

A diamine spacer was next attached to the silica particles. In this step, 5 ml of 0.1 *M* bicarbonate buffer (pH 8) was added to the support and 400 μl of 1,4-diaminobutane (3.97 mmol) were introduced, and the mixture was adjusted to pH 8.5 (ref. 7). The suspension was shaken overnight. During the reaction a bright yellow color developed confirming the presence of Schiff's base. The Schiff's base was reduced to a secondary amine with sodium borohydride (Fig. 2); typically, 100 mg of sodium borohydride were dissolved in 1 ml of deionized/distilled water[8]. Approximately, 1/3

of the sodium borohydride solution was added to the amine support at time $t = 0$, $t = 20$, and $t = 40$ min. At $t = 60$ min the reduced support material was filtered and washed with 2 *M* potassium chloride solution and water.

In the next step, the peptide substrates were bound to the support. A carbodiimide was utilized as the coupling catalyst. Threefold excess of carbodiimide and Boc-L.Phe compared to the amine group is often reported as a suitable reaction ratio[9]. If about 200 μmol/g of aminospacer existed on the support, 0.9 mmol (*i.e.*, 0.6 mmol/g) were added; therefore, 238 mg of Boc-L-Phe were dissolved in 6 ml of methanol with 2 ml of water, and the peptide solution was combined with the support. Subsequently, 172 mg of EDC, dissolved in 1 ml of water, were added dropwise. The acidity of the reaction mixture was adjusted to a pH between 5 and 6. The mixture was left on a shaker overnight. The next day the derivatized silica was filtered and washed with methanol and water.

The peptides on the external surface of the silica particles were then removed by treatment with α-chymotrypsin as illustrated in Fig. 2. For the enzyme cleavage procedure, 25 mg of chymotrypsin were dissolved in 1 ml of 0.001 *M* hydrochloric acid and diluted to 10 ml with 0.08 *M* Tris buffer in 0.1 *M* calcium chloride solution (pH 7.8). The reaction was allowed to proceed for several hours whereupon another 25-mg dose of chymotrypsin was added. The suspension was then shaken overnight. The enzyme-treated silica support was filtered and sequentially rinsed with 0.1 *M* acetic acid at pH 4.0, methanol and water.

Quantification of substrate cleaved from the support

Two methods were used to demonstrate that α-chymotrypsin had cleaved the substrates from the silica support. In the first method, a filtrate of the reaction mixture was analyzed for the presence of blocked peptide (or blocked amino acid). As Fig. 2 illustrates the molecules cleaved off by the enzyme are the same as the original substrate peptide; therefore, Boc-L-Phe should be liberated into the surrounding solution from an Boc-L-Phe-modified support upon enzyme treatment.

Before chymotrypsin addition, the silica particles are exhaustively washed with methanol, tetrahydrofuran and water to ensure that no surface-adsorbed Boc-L-Phe was present on the support before cleavage. A reversed-phase chromatographic separation of components found in this final wash is illustrated in Fig. 3a. Standard Boc-L-Phe, dissolved in the mobile phase and injected under the same chromatographic conditions is illustrated in Fig. 3c. Comparison of the two chromatograms demonstrates that no Boc-L-Phe remained in the final wash solution.

The chymotrypsin was then added to the derivatized support to promote cleavage. After completion of the enzyme reaction, the support particulates were isolated by filtration with 0.22-μm filters. The filtrate was then subjected to ultrafiltration with Amicon YM10 membranes, having a 10 000-dalton cutoff, to remove the chymotrypsin, which otherwise would damage the ODS HPLC column. The ultrafiltrate was then injected onto the reversed-phase HPLC system. A typical chromatogram is illustrated in Fig. 3b. The peak due to the Boc-L-Phe cleaved from the silica support is evident.

In the second method, both the non-enzyme-treated and the enzyme-treated supports were hydrolyzed with strong acid. The amide bonds are severed as indicated in Fig. 4a. In this procedure, about 100 mg of support, dried in vacuum over phos-

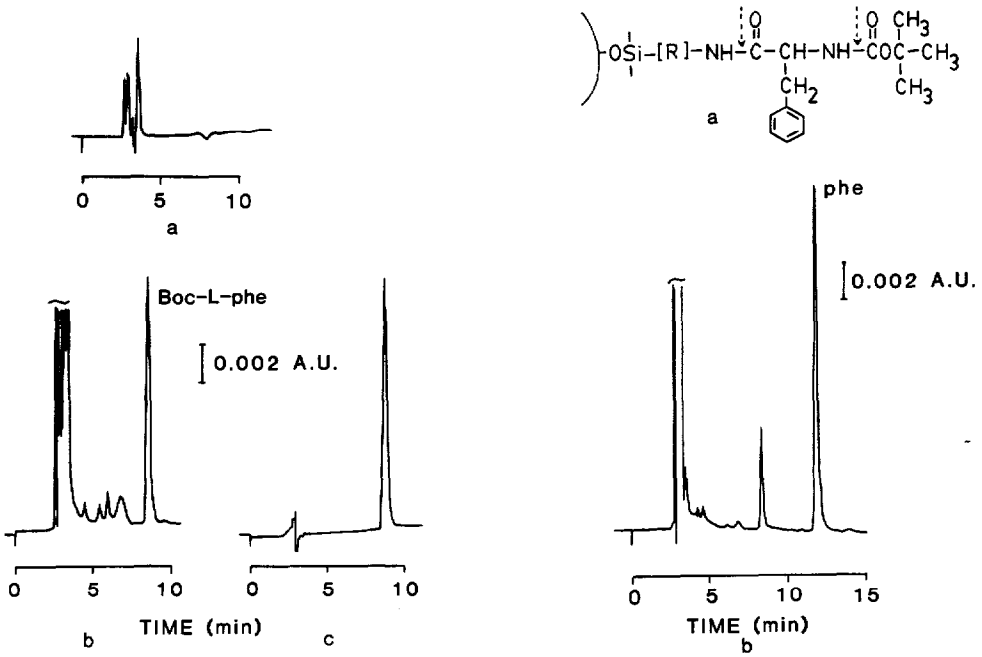


Fig. 3. Reversed-phase chromatographic separation of enzyme-cleaved Boc-L-Phe on 25 cm × 4.6 mm I.D. ODS column with acetonitrile–methanol–0.1 *M* orthophosphate buffer, pH 6.0 (20:33:47) mobile phase. Flow-rate = 1.0 ml/min; UV detection at 254 nm. (a) Last wash before enzyme treatment; (b) liberated Boc-L-Phe in ultrafiltrate of reaction mixture after enzyme treatment; (c) pure Boc-L-Phe standard.

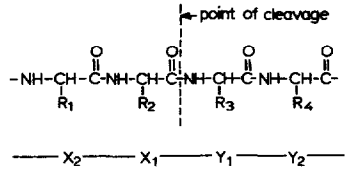
Fig. 4. Reversed-phase chromatographic separation of phenylalanine from strong acid hydrolysis of Boc-L-Phe derivatized support. (a) Bound Boc-L-Phe; arrows indicate hydrolysis points; (b) chromatographic separation; column and detection as in Fig. 3; mobile phase consisted of methanol–0.1 *M* orthophosphate buffer, pH 2.9 (10:90).

phorus pentoxide, were accurately weighed and hydrolyzed with 6 *M* hydrochloric acid for 24 h. Upon acid hydrolysis all of the derivatized peptide supports yielded phenylalanine. The phenylpropionic acid-modified silica yielded phenylpropionic acid. The hydrolyzed mixtures were diluted with buffer, filtered through a 0.22-μm filter, and injected onto the reversed-phase HPLC system. The chromatographic mobile phase consisted of methanol–0.1 *M* orthophosphate, pH 2.9 (10:90) as optimized by Mönch and Dehnen[10]. A chromatogram of a hydrolysis filtrate from the Boc-L-Phe support demonstrates the presence of phenylalanine (Fig. 4b). In this second method, quantification of the phenylalanine, acid hydrolyzed from both the non-enzyme-treated and enzyme-treated supports, yielded the amount of enzyme cleaved substrate by difference.

RESULTS AND DISCUSSION

α-Chymotrypsin preferentially catalyzes the cleavage of peptide bonds involving L-isomers of tyrosine, phenylalanine and tryptophan. The primary criteria for

cleavage is that an aromatic side group R_2 be immediately adjacent to the susceptible bond on the N-terminal side of the peptide, as illustrated below.



In homogeneous solutions secondary substrate effects can alter the reactivity of chymotrypsin. Chymotrypsin, being an endopeptidase, does not tolerate any free carboxylic acids or free amines close to the active site[11]; therefore, if a short peptide is used as the substrate, the two ends must be blocked with uncharged groups. As shown in Fig. 1, all substrates were attached via an amine spacer and the N-terminal amino acids blocked.

Amino acid Y_1 adjacent to the cleavage point on the carboxylic acid side must differ from the amino acid X_1 on the amine terminal side. A bulky amino acid in the R_3 position decreases reactivity[12]. A D-amino acid in the R_3 position, which would direct its R group towards the enzyme instead of its α-hydrogen as would an L-amino acid, leads to complete inactivity. Also, a proline residue in the R_3 position yields inactivity. It becomes obvious that a small amino acid side group in the R_3 position is advantageous. Glycine as the R_3 amino acid or simply a $-CH_2-$ moiety adjacent to the susceptible bond renders an enhanced activity to the chymotrypsin. It has been demonstrated that a tetrapeptide bound to a polyacrylamide polymer with a $-CH_2-$ group to this side of the susceptible bond could be cleaved by chymotrypsin with high yields[13].

In the cleavage of peptides drom a solid phase, the length of the spacer arm is also critical. If the spacer is too short, steric hinderance prevents the enzyme from conforming to the reaction site. Fischer *et al.*[14] have demonstrated that the degree of hydrolysis of L-Phe-4-nitroanilide bound to agarose on treatment with chymotrypsin was less without a spacer and greater with a six-carbon-chain spacer. On the other hand, if the spacer is too long, non-specific effects become pronounced. A spacer of 17–20 Å in length appears the most suitable for the enzyme cleavage of substrates from silica supports.

With the above conditions fulfilled, a Boc-L-Phe-modified support was subjected to treatment with α-chymotrypsin. The chymotrypsin cleaved the blocked amino acid from silica surface demonstrating the potential for removal of peptides from solid silica surfaces. Once it had been established that chymotrypsin could cleave a substrate from a silica surface, the degree of such heterogeneous hydrolysis with selected substrates was studied. In homogeneous solutions secondary substrate effects, such as the types of amino acids adjacent to the susceptible bond, have significant affect on reactivity. Each substrate (Fig. 1) was selected for its demonstrated reaction characteristics in homogeneous solution. For example, it has been suggested that the reactivity of chymotrypsin increases with longer-chain substrates[15]. This is especially true when additional amino acids (*e.g.*, X_2) are present on the amine terminal side of the cleavage point[16]. It has been proposed that in solution phase a dipeptide provides a greater area than a single amino acid for association to the

TABLE I

QUANTIFICATION OF BOUND SUBSTRATES BEFORE AND AFTER ENZYME TREATMENT

Bound substrate	*Untreated (μmol Phe/ g support)*	*Enzyme treated (μmol Phe/ g support)*	*Amount cleaved off*	
			μmol Phe/ g support	*Per cent cleaved*
Cbz-L-Val-L-Phe	103	96	7	7
Boc-L-Phe	165	130	35	21
Acetyl-L-Phe	140	108	32	23
Bz-L-Phe	100	92	8	8
Phenylpropionic acid	150	137	13	9

enzyme. In addition, if the adjacent amino acid in position X_2 is a hydrophobic amino acid (*e.g.*, valine) the reactivity is increased[17]. Because of the valine in position X_2, the Cbz-L-Val-L-Phe was selected as a substrate to evaluate if this phenomenon could also be observed at a heterogeneous silica interface.

The remaining substrates were selected for various reasons.Acetyl-L-Phe (Fig. 1c) is commonly selected for kinetic studies. Small peptides with N-terminal amino acids blocked by an acetyl group have been reported to have high reactivity towards alpha chymotrypsin. Bz-L-Phe (Fig. 1d) was chosen because of its similarity to the substrate, benzoyl-L-tyrosine ethyl ester (BTEE), currently used by most suppliers to measure α-chymotrypsin activity. This substrate is very susceptible to chymotrypsin suggesting that an L-phenylalanine with a benzoyl group on the N-terminal of its chain would also be hydrolyzed readily.

Chymotrypsin also promotes cleavage of synthetic amides and esters. Even when a synthetic molecule is hydrolyzed by chymotrypsin the suseptible peptide bond requires an aromatic or hydrophobic group. Reports describing cleavage of synthetic substrates connected to soft gels can be found[18]. In this case the synthetic substrate group was always at the end of the polymer chain. Here phenylpropionic acid (Fig. 1e) was selected as a synthetic substrate.

Table I indicates the ability of α-chymotrypsin to cleave the four peptide substrates and the synthetic substrate from the silica matrix. Boc-L-Phe and acetyl-L-Phe are both liberated in high yields. In reviewing the per cent cleavage, recall that the nominal pore size of the silica particles was small (40 Å) which prevents chymotrypsin from entering the internal surface. Only substrate molecules bound to the external surface are cleaved off. Since the internal surface of porous silica particle is always much larger than the external surface[19], 23% cleavage is very high and suggests that in fact all bound substrate on the exposed surface was removed.

Neither Cbz-L-Val-L-Phe nor Bz-L-Phe were removed from the silica surface in substantial yields. Both substrates included an aromatic N-terminal blocking group. This aromatic group may, through steric or electronic interactions, prevent chymotrypsin from reaching the susceptible peptide bond which is located between the blocking group and the silica surface. The secondary substituent effects which enhance activity in a homogeneous solution phase appear to be inoperable when the hydrolytic cleavage is conducted at the heterogeneous silica interface.

In conclusion, it can be said that α-chymotrypsin does not have the same reac-

tivity for certain substrates when connected to silica surfaces as in free solutions. The two molecules Cbz-L-Val-L-Phe and Bz-L-Phe which are easily hydrolyzed by the enzyme in homogeneous solution are not substantially cleaved when attached to silica by the described method. The synthetic substrate phenylpropionic acid is cleaved off in a low yield as well. An attached chain to the silica surface containing Boc-L-Phe or acetyl-L-Phe is, however, easily hydrolyzed by α-chymotrypsin.

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