2218991

ENZYMATIC MEMBRANE METHOD FOR THE SYNTHESIS OF PEPTIDES AND THE SEPARATION OF OPTICAL ISOMERS

Technical Field

The present invention relates generally to an enzymatic method for the synthesis and separation of peptides employing a membrane permeable to uncharged peptides but impermeable to charged molecules; and, more particularly, to the simultaneous synthesis and purification of L,L-dipeptides, and its application to the preparation of L-aspartyl-L-phenylalanine methyl ester (aspartame).

Background Art

The use of proteolytic enzymes as condensation catalysts for the stereospecific coupling of two L-amino acids to yield L, L-peptides is known since the early days of protein chemistry. As early as 1909 Mohr and Strohschein described the formation of the water-insoluble dipeptide Bz-Leu-Leu-NH_2 by reacting Bz-Leu-OH and H-Leu-NH_2 in the presence of the protein degrading enzyme papain. E. Mohr and F. Strohschein, Ber 42, 2521 (1909). The Mohr and Strohschein-type reaction is possible only between those amino acids that form peptide bonds that are susceptible to cleavage by the papain or other enzyme used. Indeed, the condensing reaction's equilibrium between the amino acid reactants and peptide product is largely displaced towards the reacting amino acids. Nevertheless, the condensing reactant can be driven to completion by mass action if, e.g., the dipeptide product is poorly soluble and precipitates out of the reaction phase.

Due to the commercial importance of certain peptides and the fact that enzymes are known to catalyze peptide formation under mild conditions there has been a great deal of research done on the enzymatic synthesis of peptides particularly simple dipeptides. K. Oyama and

K. Kihara, Kagaku Sosetsu 35, 195 (1982); K. Oyama and K. Kihara, ChemTech. 14, 100 (1984).

The process for enzymatic synthesis of the peptide derivative aspartame, described in U.S. Patent 4,165,311, hereinafter the '311 process, involves the thermolysin-catalyzed condensation of N-carbobenzoxy-L-aspartic acid with D,L-phenylalanine methyl ester and precipitation of an intermediary complex, D-phenylalanine methyl ester salt of N-carbobenzoxy-aspartame, to drive the reaction to the peptide product side. Further processing of this intermediary complex allows for the recovery of D-phenylalanine methyl ester, that may be recycled after racemization, and of the N-carbobenzoxy-aspartame derivative which can be converted to aspartame by elimination of the N-carbobenzoxy protecting group. The '311 process must be practiced on a batch basis which is cumbersome and complicates the recovery of enzyme. Also see: K. Oyama, S. Irino, T. Harada and N. Hagi, Ann. N.Y. Acad. Sci. 434, 95 (1985). Willi Kullmann, Enzymatic Peptide Synthesis (1987).

The N-carbobenzoxy protecting group plays an essential role in the '311 process by: fulfilling the structural requirement imposed by the active site of thermolysin; and by contributing to the insolubility of the intermediary complex thereby increasing the yield of the reaction. Elimination of the N-carbobenzoxy protecting group from the aspartame derivative must be effected under mild conditions, e.g., catalytic hydrogenation, to prevent cleavage of the methyl ester function. Catalytic hydrogenation involves the inconvenience of handling hydrogen gas on a large scale.

Alternative approaches to driving enzymatic condensation reactions to completion have also been described in the chemical literature. For example, the use of organic solvents as reaction media has been found effective for increasing the peptide product yields; although, the concomitant decrease in enzyme stability has

precluded its practice on a large scale. K. Oyama, S. Nishimura, Y. Nonaka, K. Kihara and T. Hashimoto, J. Org. Chem. 46, 5241 (1981); H. Ooshima, H. Mori and Y. Harano, Biotechnology Letters 7, 789 (1985); K. Nakanishi, T. Kamikubo and R. Matsuno, Biotechnology 3, 459 (1985).

In view of the above-noted difficulties in the practice of prior art methods for enzymatic synthesis of peptides, particularly, the requirements for precipitation of an intermediary complex and handling of dangerous reagents,it would be desirable to provide an improved process that avoids these difficulties and that safely provides effective yields without rapid deactivation of the enzyme catalysts.

Disclosure of Invention

The present invention provides a method for the enzymatic synthesis of peptides comprising the steps of:

enzymatically condensing first and second amino acid compounds in an aqueous initial reaction mixture to form an uncharged peptide.

transporting the uncharged peptide into an aqueous reaction mixture by osmosis across a membrane that will not transport substantial amounts of the amino acid compounds; and

converting the transported uncharged peptide to a form that cannot be retransported across the membrane to the initial reaction mixture.

The transported compound, converted to a form that is not retransported across the membrane, can be removed from the second reaction mixture. Also, first and second amino acid compounds copermeating the membrane with the uncharged compounds into the second reaction mixture can be separated from the second reaction

mixture and optionally returned to the initial reaction mixture.

A preferred aspect of the invention provides a process for the enzymatic synthesis of peptide derivatives, such as aspartame and their analogs, comprising the steps of condensing a N-acyl-β-substituted-L- aspartic acid including an α-carboxylate group with a phenylalanine lower alkyl ester including an α-ammonium group in an aqueous reaction mixture including a condensation enzyme, to from N-acyl-L-aspartyl (β-substituted) -L-phenylalanine lower alkyl ester (i.e. 1-6 carbons), an uncharged peptide; transporting the uncharged peptide from the aqueous reaction mixture across an ion rejection membrane into a product mixture by osmosis; and converting the uncharged peptide in the product mixture to a species that cannot back-diffuse across the membrane.

The present invention also provides a method for the enzymatic resolution of racemic carboxylic acid compounds comprising the steps of; hydrolyzing a racemic carboxylic acid ester in an aqueous reaction mixture including a hydrolyzing enzyme to form a charged enantiomeric compound and an uncharged enantiomeric ester compound in the aqueous reaction mixture; and transporting the uncharged enantiomeric ester compound from the aqueous reaction mixture across an ion rejection membrane into a product mixture thus effecting an enantiomeric resolution.

The racemic carboxylic acid ester compound is preferably an D, L-amino acid ester compound, the charged enantiomeric compound is preferably a L-amino acid compound and the uncharged enantiomeric compound is the corresponding D-amino acid ester compound. The uncharged D-amino acid compound in the product mixture can be accumulated and can be recovered by conventional means. The method for the enzymatic resolution of amino

acid compounds can be combined with the other methods discussed above.

It is an object of the present invention to provide a process for the enzymatic synthesis of peptides which provides for simultaneous synthesis and purification of the peptide product.

It is another object of the present invention to provide a process for the safe, economical and efficient synthesis and purification of peptides and derivatives thereof, particularly aspartame.

It is another object of the present invention to provide an economical process for the enzymatic synthesis of peptides that provides for the efficient use of enzyme and the means to effect the synthesis on a continuous basis.

It is yet another object of the present invention to provide a process particularly adapted to the enzymatic synthesis of aspartame and its derivatives with D, L-phenylalanine and N- protected-β-substituted-L-aspartate in substantially quantitative yield.

Brief Description of Drawings

The foregoing and other objects and advantages are attained by the invention, as will be apparent from the following detailed description taken in conjunction with the accompanying drawings, wherein:

Figure 1 is a schematic illustration of the enzymatic synthesis of asparatame in accordance with the present invention.

Figure 2 is a schematic representation of an apparatus for practicing the process of the present invention.

Figure 3 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Examples 1 and 2.

Figure 4 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 4.

Figure 5 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 5.

Figure 6 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 6.

Figure 7 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 7.

Figure 8 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 8.

Figure 9 is a schematic representation of an apparatus for practicing the present invention which illustrates the vessels on the product side as described in Example 9.

Figure 10 is a graph illustrating the quantity of product (aspartame derivative) formed over time in Example 9 with and without the utilization of an ion exchange resin.

Best Modes for Carrying Out the Invention

The invention disclosed herein provides a procedure for driving to completion the enzymatic synthesis of peptides in an aqueous reaction mixture by separating the uncharged peptide intermediate, or derivative thereof, from the reaction mixture by means of a membrane that selectively transports the uncharged peptide out of the

reaction mixture into a product mixture. Because the membrane is substantially impermeable to the reactants (charged molecules) removal of the peptide intermediate from the reaction mixture causes a decrease of peptide concentration at equilibrium that pushes the reaction toward completion by mass action.

The membranes most useful in the practice of the present invention are Immobilized Liquid Membranes (ILM) comprising a nonpolar liquid embedded in microporous support material preferably a polymeric sheet that is substantially impervious to the enzymes, reactants and products. Hydrophobic polymers such as polypropylene are preferred support materials. ILM modules can be produced utilizing polypropylene hollow fibers. Celgard, a registered trademark of the Celanese Corporation, 1211 Avenue of the Americas, New York, N.Y. 10036 and sold by Celanese Fibers Marketing Corporation, Charlotte, N.C., is an example of commercially available hollow fibers of polypropylene. Potting compounds known in the art and polyvinyl chloride pipe or tubing may optionally be utilized in fabricating an ILM module. Another polymer for fabricating the microporous support material is TEFLON, a trademark of E. I. DuPont de Nemours & Co. for fluorinated hydrocarbon polymers. A typical microporous support is GORE-TEX, a trademark of W. C. Gore & Associates, Inc.

The micropores pass through the support material and should be sized so that an immobilized liquid will be held therein by capillarity and will not escape therefrom when subjected to, e.g., pressure differentials across the membrane or other ordinary reaction conditions. Subject to the foregoing limitations is advantageous to maximize the contact area between the immobilized liquid and reaction mixture to maximize the rate of transfer (flux) of the uncharged peptide product across the membrane. It will be appreciated that the preferred pore size will vary depending on the properties of the particular immobilized

liquid, reactants employed, products produced, and like factors; and further, that the optimum pore size can be determined empirically by those skilled in the art. A useful discussion of pore size selection is found in U.S. Patent No. 4,174,374. The use and preparation of immobilized liquid membranes are described in the following references, S. L. Matson, J. A. Quinn, Coupling Separation and Enrichment to Chemical Conversion in a Membrane Reactor, paper presented at the AICHE Annual Meeting, New Orleans, Louisiana (November 8-12, 1981) and S. L. Matson and J. A. Quinn, Membrane Reactors in Bioprocessing, Ann. N.Y. Acad. Sci. 469, 152 (1986).

The immobilized liquid held in the microporous support by capillarity should be water immiscible and a good solvent for the uncharged peptide product which must be transported across the membrane (diffused) at a reasonable rate, i.e., good transport characteristics/high flux; while, charged or ionized molecules on both the reaction side and product side of the membrane are, for the most part, not transported across the membrane in either direction, i.e, good selectivity/ion rejection.

Selection of the best combination of support material and immobilized liquid for use in an enzymatic synthesis of a peptide in accordance with the present invention will depend, in part, on the nature of the particular reactants employed, products desired and solvents in the system.

The generally preferred immobilized liquids for the practice of the present invention include water-immiscible organic solvents, such as alcohols of 6 to 20 carbons, branched and unbranched, for example, n-decanol, n-dodecanol, and n-hexadecanol. Also preferred are N,N-diethyl-dodecanamide, dodecane and 2-undecanone. Mixtures of the above may also be used.

Another type of membrane useful in the practice of the invention comprises hydrophobic solid films made of

organic polymers such as polyvinyl chloride or the like. The preparation of these polymer membranes is well described in the literature, for example, O. J. Sweeting, Editor, Science and Technology of Polymer Films, Interscience, New York (1968), while extensive application of such membranes to the separation of gases and liquids are discussed in S. T. Hwang and K. Kammermeyer, Membranes in Separations, Techniques of Chemistry, Vol. VII, (A. Weissberger, editor) John Wiley & Sons, Inc., New York (1975).

A preferred embodiment of the invention employs a membrane reactor/separator system which provides an aqueous reaction mixture or phase circulating in contact with one side of an ILM membrane and a product aqueous phase or mixture circulating countercurrently at the opposite surface of the membrane. S. L. Matson and J. A. Quinn, Ann. N.Y. Acad. Sci. 469, 152 (1986). The pH and temperature of the reaction and product phases are maintained at a value that keeps the reactants in a form that minimizes their transport across the membrane at pH's between about 4.0 and 9.0. Transport of the uncharged peptide intermediate from the reaction phase to the product phase is driven by the concentration gradient across the membrane created by increasing neutral or uncharged peptide concentration in the reaction phase. The transport activity or flux across the membrane can be significantly enhanced by the simultaneous, irreversible conversion of the transported peptide, in the product phase, to a species that cannot back-diffuse. For example, the latter conversion may result in the formation of a polar peptide that cannot back-diffuse and thus supplement the driving force to achieve completion of the coupling reaction. An example of a membrane reactor/separator that could be adapted for the practice of the present invention is found in U.S. Patent No. 4,187,086.

Available alternative membrane reactor/separator configurations that could be adapted to practice of the present invention include the hollow fiber modules described in U.K. Patent Application 2,047,564 A, and conventional plate and frame-type filter devices well known in the art.

In addition to selective transport of the uncharged peptide the membrane provides a barrier between the reaction phase and product phase that prevents undesirable mixing of, and reactions between, the components of each phase.

In a preferred membrane reactor/separator configured in accordance with the present invention, the chemical equilibrium between the reactants is actually "pulled" across the membrane by conducting an irreversible conversion of the transported uncharged peptide, to a membrane impermeable species, on the product side of the membrane. This type of membrane reactor/separator employs a coupled two enzyme (E^1 and E^2) process of the general type:

$$\underline{A}^+ + \underline{B}^- \overset{E^1}{\underset{}{\rightleftarrows}} \underline{C} \overset{E^2}{\rightarrow} \underline{D}^{\pm}$$

The charged reactants A and B are amino acids and/or small peptides which are condensed with the aid of peptide forming enzyme E^1 to form the uncharged intermediary peptide C which is selectively transported across the membrane to the product side. It is understood that reactive functional groups in the reactants that do not participate in the desired reaction may be protected or blocked, where necessary, to prevent undesirable side reactions and/or changes in the products. On the product side of the membrane uncharged peptide C is converted to charged peptide D which cannot back-diffuse across the membrane, causing the chemical equilibrium in the reaction mixture to shift toward the production of more C.

This concept is illustrated in Figure 1, for the specific case of the enzymatic condensation of D,L-phenylalanine methyl ester with (N-and ß-protected) N-formyl-ß-benzyl-L-aspartate, in the presence of thermolysin at about pH 5.5.

In the reaction scheme illustrated in Figure 1 the reactant $\underline{A}^+$ is D, L-phenylalanine methyl ester and $\underline{B}^-$ is N-formyl-ß-benzyl-L-aspartate. The reactants are condensed on the reaction side of the membrane by the enzyme E^1 thermolysin forming the uncharged peptide C. The pH is selected to maintain the reactants in their charged state and thus minimize their diffusion across the membrane along with uncharged peptide C.

Although the chemical equilibrium for the condensation reaction largely favors the reactant $\underline{A}^+$ and $\underline{B}^-$ species, diffusion of the uncharged peptide product C across the membrane to the product side requires the constant production of C to maintain the chemical equilibrium on the reaction side of the membrane.

On the product side on the membrane an esterase enzyme E^2 quickly and irreversibly converts the uncharged peptide C diffused across the membrane to charged peptide D which cannot back-diffuse to the reaction side. Thus the chemical equilibrium on the reaction side is effectively "pulled" across the membrane and toward the production of uncharged product C. Thereafter, the peptide D is converted to aspartame by acid hydrolysis, which removes the formyl and benzyl protecting groups, followed by C-terminal esterification with methanol.

The foregoing method may be practiced in a countercurrent flow membrane reactor/separator 10, as shown in Figure 2, operating under controlled conditions of pH and temperature, so that a reaction mixture comprising a N-acyl-ß-substituted-L-aspartic acid, e.g., N-formyl-ß-benzyl-L-aspartate, having a free α-carboxylate group (electronegative species) which is allowed to condense with a reactant, e.g, D, L-phenylalanine methyl

ester, having a protonated free α-amino group (electropositive species), under the catalytic action of proteases active in the pH range of 4.0-9.0, to yield a fully protected L-aspartyl-L-phenylalanine dipeptide bearing no ionized groups (electroneutral species). On the reaction side of the membrane, the reaction mixture is circulated from reactor tank 12 aided by pump 14, through feed-in conduit 16 through separator 18 to feed-out conduit 20 which returns to reactor tank 12. On the product side of the membrane a product mixture or sweep including fully protected uncharged peptide, e.g., N-formyl-β-benzyl- L-aspartyl-L-phenylalanine methyl ester transported across the membrane, is circulated from product reactor tank 22, aided by pump 24, through product sweep in-conduit 26, through the product side of separator 18, to product sweep out-conduit 30. This product mixture includes a second enzyme E^2 an esterase, or other suitable reagent, that can cleave at least one of the protected groups borne by the uncharged peptide, thus generating an electrocharged species that cannot escape the sweep stream by back-diffusing through the membrane. If an esterase is utilized, a preferred esterase will have proteolytic activity and a preferred pH range of from 6.0 to 9.0. Aminoacylase I, α-chymotrypsin and subtilisin A are examples of esterases considered useful in the present invention, the preferred pH value for the first of these being about 7.0.

The charged product may be periodically discharged and/or continuously removed from the product phase by conventional means such as ion exchange resins, and the remaining effluent may be recycled through the system. The resulting product bound to the ion-exchange resin may be desorbed and recovered using conventional procedures.

The selection of appropriate deprotection reagent(s) is determined by the chemical nature of the

protecting groups used on the reactants, such as N-protected-L-aspartic acid, and as indicated above the choice of protecting groups is in turn dictated by structural constraints imposed by the active site of the condensing enzyme.

In general, the enzymes useful in the practice of the present invention are proteolytic enzymes, sometimes called proteases, which may be divided into two groups. The more commonly used are endopeptidases, which only cleave inner linkages, and exopeptidases, which only cleave terminal linkages. Useful enzymes include serine proteinases, (EC 3.4.21) such as chymotrypsin, trypsin, subtilisin BNP' and Achromobacter protease; thiol proteinases (EC 3.4.22), such as papain; carboxyl proteinases (EC 3.4.23), such as pepsin; and metalloproteinases (EC 3.4.24), such as thermolysin, prolisin, Tacynasen N (St. caespitosus) and Dispase. Conveniently such enzymes are used in the pH range 4.0 to 9.0, preferably 4.0 to 6.0. Binding of the enzymes to insoluble supports, following procedures well known to practitioners of the art, may be incorporated to the practice of this invention; and although binding the enzymes is not an essential step, it may be desirable in certain applications. When an immobilized enzyme is used, the pH of the reaction mixture is advantageously re-adjusted to about 5.0 prior to transporting the uncharged peptide from the reaction mixture, for example across an ion rejection membrane. Among the many proteases potentially useful for the practice of this invention, thermolysin (EC. 3.4.24) is the condensing enzyme most preferred because of its remarkable thermostability, wide availability, low cost and broad useful pH range from 5.0 to 9.0. The preferred pH for thermolysin is about 7.0. Other preferred proteases include pepsin and penicillopepsin [T. Hofmann and R. Shaw, Biochim. Biophys. 92, 543 (1964)] and the thermostable protease from Penicillium duponti [S. Emi,

D. V. Myers and G.A. Iacobucci, Biochemistry 15, 842 (1976)]. They would be expected to function at about pH 5.5., exhibit good stability at such pHs, and do not require the presence of Zn++ and Ca++ ions to maintain their activity.

The practical realization of enantiomeric selectivity that is, in the case described above, production of only the L,L isomer of the peptide C, is directly related to the enzyme selected, optimal functioning of the membrane,

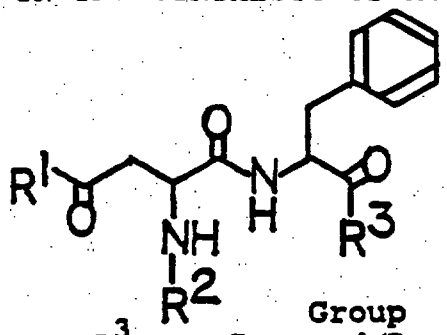
chemical nature of the support material and pH of the aqueous reaction phase.

One preferred membrane for practicing the above-described specific method is a microporous polypropylene support material including a mixture of n-hexadecanol and n-dodecane immobilized therein. This membrane is available from Bend Research, Inc., 64550 Research Road, Bend, Oregon 97701, U.S.A. under the tradename/designation "Type 1 Hollow Fiber Selective Dialysis Membrane" and is preferred with the reactions of Examples 1-4. Another preferred membrane utilizes Celgard Type 2400 polypropylene hollow fibers (Celgard is a registered trademark of the Celanese Corporation, 1211 Avenue of the Americas, New York, N.Y. 10036. Celgard can be obtained from Celanese Fibers Marketing Corporation, Charlotte, N.C.) as the microporous support material including a mixture of 30% w/v N,N-diethyl-dodecanamide in dodecane as the water-immiscible organic liquid immobilized by capillarity in pores of a microporous sheet of Celgard. This membrane was obtained from Bend Research, Inc., 64550 Research Road, Bend, Oregon 97701, U.S.A. under the tradename/designation "Type 2 Hollow Fiber Selective Dialysis Membrane" and is preferred with the reactions of Examples 5-9. Celgard Type 2400 polypropylene hollow fibers having a pore size of 0.025 - 0.050 μm and a wall thickness of 25 μm were utilized in the Type 2 Hollow Fiber Selective Dialysis Membrane of Bend Research, Inc. When operated at pHs of about 5.5, these membranes exhibit a high selectivity, for example, when practicing the process of Figure 1, selectivity in the range of about 500:1 (w/w) in favor of the uncharged peptide species have been measured. That is, 500 milligrams of the uncharged peptide ($\underline{C}$) are transported across the membrane for each milligram of charged reactant ($\underline{A}^+$ or $\underline{B}^-$) transported.

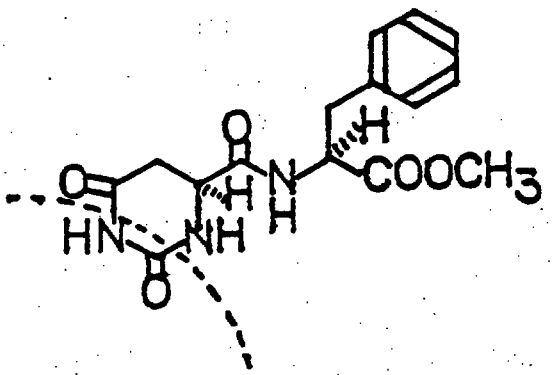
As mentioned above, for the application of the present invention it will be necessary, or desirable, to

block or protect various functional groups on the reactants and products to prevent undesirable side reactions that could prevent production of the desired product and/or reduce its yield and to suppress electro charges in intermediate peptide. Table I below lists a series of selected combinations of protecting groups and deprotection conditions useful in connection with the practice of this invention.

TABLE I. PROTECTING GROUPS AND DEPROTECTION REAGENTS USEFUL IN THE SYNTHESIS OF ASPARTAME (APM).



R^1	R^2	R^3	Group Removed/Reagent	Product
ϕCH_2O-	ϕCH_2OCO-	CH_3O-	R^1, $R^2/(Pd)H_2$	APM
ϕCH_2O-	-CH = O	CH_3O-	R^2/H_3O^+	ß-benzyl-APM
ϕCH_2O-	-CH = O	CH_3O-	R^3/Fungal esterase	N-formyl-ß-benzyl-L-aspartyl-L phenyl-alanine
ter-BuO	-CH = O	CH_3O-	R^1,R^2/H_3O^+	APM
CH_3O-	-CH = O	CH_3O-	R^3/fungal esterase	N-formyl-isoAPM
CH_3O-	-CH = O	CH_3O-	R^3/α-chymotrypsin	N-formyl-isoAPM
NH_2-	ϕCH_2OCO-	CH_3O-	R^1/asparaginase	N-CBZ-APM
L-dihydroorotyl-L-phenylalanine methyl ester			R^1,R^2/hydantoinase	APM



CH_3O-	ϕCH_2OCO-	CH_3O-	R^3/fungal esterase	N-CBZ-isoAPM
CH_3O-	ϕCH_2CO-	CH_3O-	R^2/penicillin acylase	APM-ß-methyleste

As a result of the enantioselectivity of selected condensation enzymes and the functional discrimination exerted by the membrane, the practice of the invention using N-formyl-L-aspartyl-ß-benzyl ester and D, L-phenylalanine methyl ester could achieve a 99.8% enantiomeric resolution of the racemic phenylalanine methyl ester reactant, the L-enantiomer appearing as N-formyl-L-aspartyl(ß-benzyl)-L-phenylalanine methyl ester, an aspartame derivative, with the unreacted D-enantiomer remaining in the reaction phase.

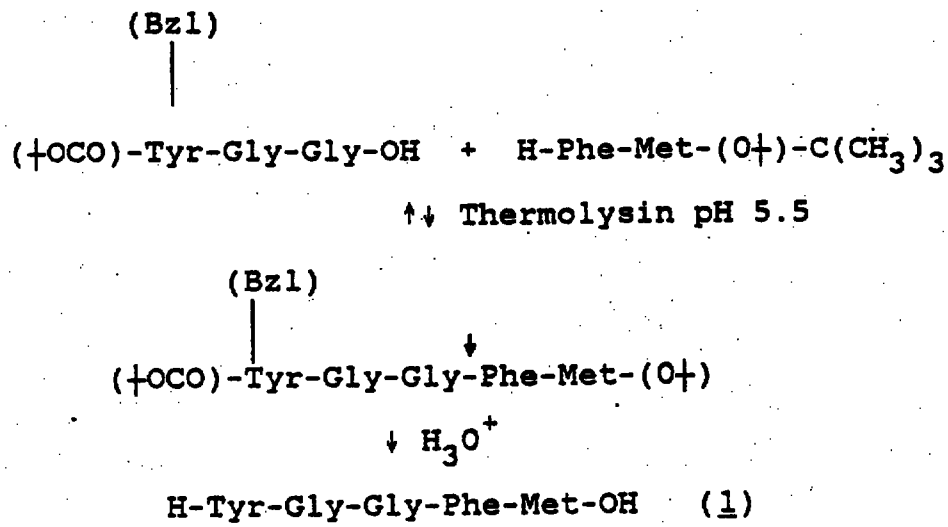
The D-phenylalanine methyl ester remaining in the reaction phase may be recovered therefrom, re-racemized to the D,L-stage, and recycled into the feedstock. This is an advantage common to many processes employing racemic amino acid reactants, e.g., the '311 process described above.

The economic advantages of the present invention derive, at least in part, from the use of a racemate feed reactant rather than a more expensive pre-resolved L-enantiomer. This advantage is made possible by the in situ optical resolution of the D,L-phenylalanine methyl ester due to: (a) the enantioselectivity of the condensing enzyme; and (b) the high selectivity of the membrane in favor of the uncharged species.

Preferred methods for the low-cost synthesis of racemic phenylalanine are those based on the utilization of benzaldehyde via 5-benzalhydantoin sometimes called the Grace process, or the catalytic carbonylation of benzyl chloride to phenylpyruvic acid, a procedure developed by Sagami Chemical Research Center, Tokyo, Japan (sometimes referred to as the Toyo Soda process of Toyo Soda Manufacturing Co., Ltd., Yamaguchi, Japan).

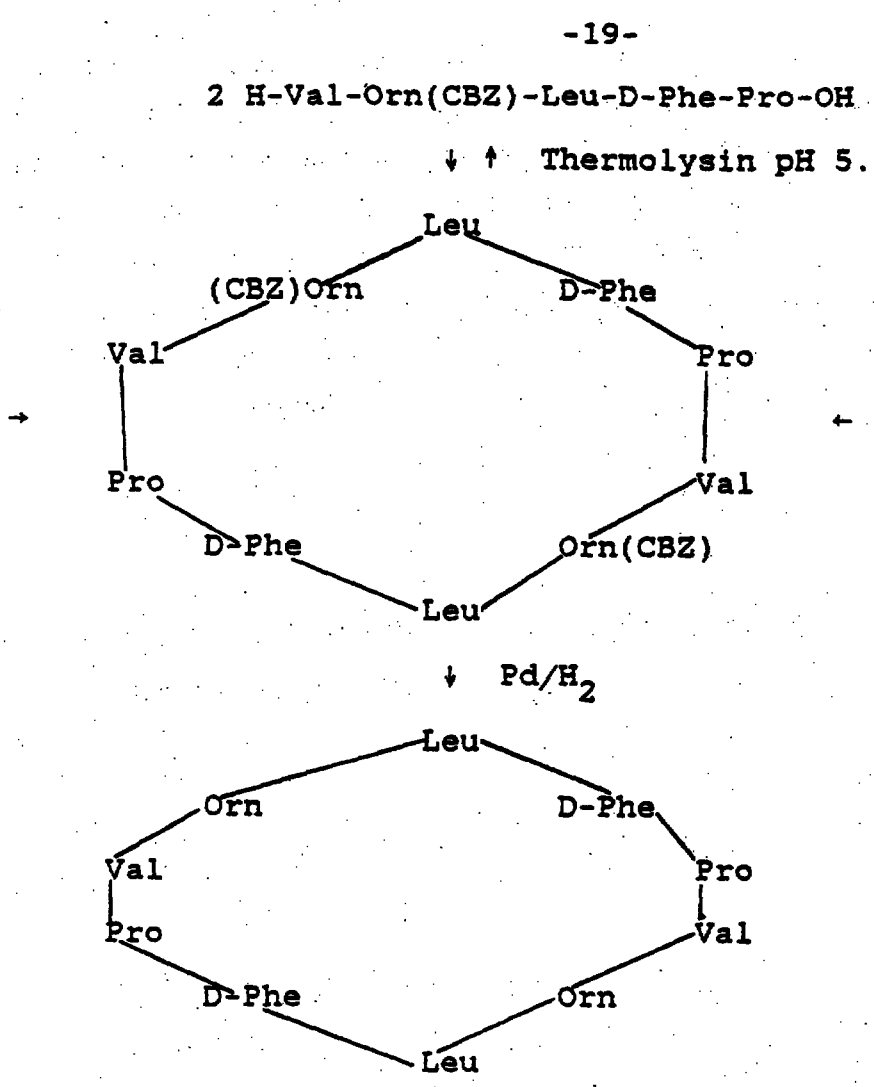
It will be appreciated by those skilled in the art that the practice of this invention is not necessarily restricted to the synthesis of peptide sweeteners, such as, aspartame or its analogs and derivatives. The invention may also be used for the synthesis of other

useful peptides, di-, tri-, tetra- and pentapeptides, or peptides of higher complexity, that are capable of diffusing through a permselective membrane at a reasonable rate. For example, considering the bond specificity of thermolysin, and assuming the presence of only one thermolysin sensitive bond in the product (indicated by the arrow in the formula below), one could synthesize met-enkephalin (1) by following scheme:



The feasibility of a stepwise total enzymatic synthesis of met-enkephalin using α-chymotrypsin and papain has been documented in the literature. See: W. Kullmann, J. Biol. Chem 255, 8234 (1980).

Another potentially useful application of the present invention is in the enzymatic synthesis of Gramicidin S (2) by the following scheme:



(2)

Various other examples of the enzymatic synthesis of useful peptide products where the present invention may find application and described in the literature are the synthesis of angiotensin, substance P, eledoisin, caerulein and Leu-enkephalin.

Those skilled in the art will appreciate that the present invention may also find application to other systems besides peptides.

For example, the use of ester hydrolases like pig liver esterase [EC 3.1.1.1] for the asymmetric resolution of prochiral dicarboxylic acid diesters into chiral monoesters is well known [C.J. Sih et al., Ann N.Y. Acad. Sci. 471, 239 (1986)]. The same enzyme can be used in

the fashion described in this invention for the resolution of racemic carboxylic acid compounds, through the selective transport of a chiral ester through the membrane and the retention of the non-reactive enantiomeric acid in the reaction phase.

The following detailed Examples are presented to further illustrate the present invention.

Example 1.

An aspartame derivative was prepared in accordance with the present invention as follows:

To a solution containing 5.02g (20 mmoles) N-formyl-L-aspartyl-ß-benzylester, 4.31 g (20 mmoles) L-phenylalanine methyl ester in 100 mL in water, adjusted to pH 5.5, was added 500 mg thermolysin enzyme (Daiwa Chem. Co., Osaka, Japan) representing a total of 8×10^5 proteolytic units.

The resulting clear reaction mixture was incubated for 15 hrs at 40°C, when the presence of insoluble dipeptide N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine methyl ester became apparent. The resulting mixture was then placed in a 200 mL vessel, connected to the reaction side, in this case tube side, of an experimental hollow-fiber separator of a Bend Research, Inc. "Type 1 Hollow Fiber Selective Dialysis Membrane" that provided 1 square foot of membrane area. The product side of the membrane (shell side of the separator) was connected to a source of aqueous (product) mixture (total volume = 200 mL) containing 500 mg of the enzyme Acylase I (EC 3.5.1.14) from Aspergillus Sp. (Sigma A 2156), at pH 7.5. This enzyme, usually described as an aminoacylase, was found to function as a C-terminal esterase, on both N-acetyl- and N-formyl-ß-benzyl aspartame.

The reaction and product mixtures were circulated at room temperature through the hollow fiber separator countercurrently at the rate of 600 mL/min, with the assistance of peristaltic pumps. The configuration of

this apparatus resembles that illustrated in Figure 2. Since both reactions (condensation and the ester hydrolysis) are protogenic, the pH in both the reaction and product mixtures drops as the process progresses. Constancy of pH, in both the reaction and product mixtures, was maintained through the use of pH stats.

The formation of N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine was monitored by HPLC. Chromatographic analysis was conducted on a Tracor Model 995 instrument along with a LDC Spectromonitor II detector set at 254 nm for the detection of the amino acids, fully protected product dipeptide, and dipeptide. The column used was a NOVA-PAK C_{18} Radial-Pak cartridge, 8mm x 10cm, housed in a Millipore Waters RCM-100 Radial Compression Module.

The mobile phase used for the detection of the fully protected dipeptide was a v/v mixture of 45% methanol (HPLC grade) 5% tetrahydrofuran (HPLC grade); and 50% of a 1% KH_2PO_4 buffer solution. For the detection of the product dipeptide, the mobile phase consisted of a v/v mixture of 40% methanol and 60% of a 1% KH_2PO_4 buffer solution (1 mL of triethylamine per liter solvent was added to minimize tailing and the pH was adjusted down to 4.3 using 80% phosphoric acid). The flow rate was kept at 1 mL/minute.

The HPLC data relating to formation of N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine is summarized in Table II below, and is expressed as the total amount (mg) of product dipeptide accumulated in the product solution as a function of time.

The value of the uncharged dipeptide concentration at equilibrium which corresponds to its saturation point in water at pH 5.5, was found to be about 0.05% at 25°C. The amount of uncharged dipeptide transported per square foot of membrane per hour was found to be about 200 mg, indicating that maintenance of the equilibrium required dissolution of insoluble dipeptide and/or dipeptide synthesis <u>de novo</u>. Almost complete dissolution of the

insoluble uncharged phase dipeptide in the reaction mixture was observed after about 5 hrs, when the reaction was stopped. A plot of the data of Table II is shown in Figure 3. The linear function indicates that the transfer of peptide across the membrane proceeds at a steady state. The observed rate of formation of product dipeptide of about 200 mg/ft^2/hr (Table II) was confirmed similar to the flux of uncharged dipeptide across the membrane (190 mg/ft^2/hr) measured at 0.05% in water as was anticipated on theoretical grounds.

At this point the described system would be expected to continue in steady state if continuous addition were made to the reaction mixture of the two amino acid reactants, at a rate of about 120 mg/hr each, to keep the system saturated in uncharged dipeptide.

In order to fully realize the membrane's selectivity the intercalation of a second membrane in series with the first membrane before the contact with the second enzyme may be necessary because the selectivity across a single membrane is lower due to the high amino acid concentration in the reaction solution.

The product solution (200 mL) was recovered, adjusted to pH 2.5 and cooled at 4°C overnight. The precipitate collected was recovered and recrystallized from MeOH:H_2O to give 307 mg of N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine $[\alpha]_D^{25°}$ = -5.6°(C=1.2; EtOH), identical (IR, 13C-NMR) to an authentic sample prepared by the batch hydrolysis of N-formyl-ß-benzyl-aspartame with Aspergillus esterase, $[\alpha]_D^{25°}$ = -5.3°(C=1.3; EtOH).

Table II.

Time (min)	Amount peptide/product solution (mg)
30	124
60	228
120	412
180	617
240	882
300	1015

Example 2.

An experiment similar to that of Example 1 was conducted, except for the use of 8.63g D,L-phenylalanine methyl ester instead of the L-enantiomer. The results are summarized in Table III. Isolation of the uncharged peptide from the 200 mL of product solution gave 310 mg of product, $[\alpha]_D^{25°}$ = -6.4°(C=1.4; EtOH), identical in all respects (IR, 13C-NMR) to an authentic sample of N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine.

Table III.

Time (min)	Amount peptide/product solution (mg)
30	166
60	258
120	475
180	686
240	996
300	1073

A plot of the data summarized in Table III (Figure 3) again showed the existence of a steady state process when the reactor was operated with D,L-phenylalanine methyl ester. The stereospecificity of thermolysin is demonstrated by the exclusive formation of the same L,L-dipeptide described in Example 1. The D-phenylalanine methyl ester retained in the tube phase (reaction mixture) did not inhibit the overall kinetics of peptide formation.

Example 3.

A mixture of 1.0 g N-formyl-ß-benzyl-L-aspartyl-L-phenylalanine, prepared in accordance with Example 1,

4.0 mL water, 4.0 mL tetrahydrofuran, and 1.0 mL conc. hydrochloric acid (12N) was heated at reflux for 9 hrs. The mixture was then cooled and the pH adjusted to 4.0 with 50% NaOH solution. The tetrahydrofuran was then removed by evaporation at < 35° and 20 mm Hg. Crystallization was completed by storage at 5°C for 1 hr, the sample then filtered, washed with 1 mL ice water, and dried <u>in vacuo</u> to give 367 mg of white solid. This material was identical to an authentic sample of aspartyl phenylalanine by HPLC and IR comparison. $[\alpha]_D^{25°}$ = +12° (C = 0.5; 0.1 N HCl in 50% MeOH).

Aspartyl phenylalanine has been converted to aspartame by treatment with methanol and hydrochloric acid, as described in G.L. Bachman and B.D. Vineyard, U.S. Patent No. 4,173,562, example #1.

<u>Example 4</u>.

An experiment similar to that of Example 1 was conducted, except for the use 5.65g (20.1 mmoles) N-carbobenzoxy-L-aspartic acid ß-methyl ester and 4.38g (20.3 mmoles) L-phenylalanine methyl ester as reactants. The amino acids were dissolved in 100 mL water, the pH of the solution adjusted to 5.5, and 500 mg of thermolysin Daiwa (8×10^5 proteolytic units) was added. The solution was preincubated for 15 hours at 40°C, when a substantial amount of N-CBZ-(ß-methyl ester)-L-asp-L-phenylalanine methyl ester was precipitated. The suspension was connected to the tube side of a "Type 1 Hollow Fiber Selective Dialysis Membrane" (Bend Research Inc.) containing 1 ft^2 of membrane surface, and the machine was operated at room temperature for 5 hours against a shell side phase of 200 mL water containing 500 mg Acylase I (Sigma) at pH 7.5. The accumulation of peptide product in shell phase was monitored by HPLC, and the results are reproduced in Table IV and Figure 4.

After 5 hours run the reaction was stopped, the shell side phase (200 mL) was recovered, adjusted to pH 2.5, and

stored overnight at 4°C. The product precipitated was collected and recrystallized from $CH_3OH:H_2O$ to yield 405 mg (86% recovery) of N-CBZ-(ß-methyl ester)-L-asp-L-phenylalanine (N-CBZ-iso-APM) $[\alpha]_D^{25°}$ = +6.0° (C = 1.1, EtOH), identical (^{13}C-NMR) to an authentic sample of N-CBZ-iso-APM, $[\alpha]_D^{25°}$ = +5.5° (C = 1.1, EtOH), prepared by chemical coupling and partial esterolysis with Acylase I.

Table IV.

Time (min)	Amount peptide/product solution (mg)
30	177
60	214
120	333
180	381
240	459
300	470

As observed before in the experiments of Examples 1 and 2, the plot of Figure 4 indicates that the accumulation of N-CBZ-iso-APM proceeded at a steady rate of about 200 mg/hr.

The conversion of N-CBZ- iso-APM to APM can be practiced under the conditions described in Example 3.

Example 5.

The aspartame derivative, N-formyl-(ß-methyl)-asp-phe was prepared in accordance with the present invention as follows: To a solution containing 6.00 g (35 mmoles) of N-formyl-(ß-methyl)-L-aspartic acid, 10.00 g (48 mmoles) L-phenyl alanine methyl ester in 100 mL water, adjusted to pH 7.0, was added 770 mg thermolysin enzyme (Daiwa Chemical Co., Osaka, Japan) representing a total of 1.2 x 10^6 proteolytic units. The resulting clear solution was incubated for 1 hr at 40°C, when HPLC analysis indicated the presence of 433.8 mg of N-formyl-(ß-methyl)-asp-phe-OMe. The solution was cooled to 25°C, the pH adjusted to 5.0, and the solution placed

in a 200 mL vessel connected to the tube side of hollow-fiber separator ("Type 2 Hollow Fiber Selective Dialysis Membrane", Bend Research Inc.) that provided 0.5 ft^2 (450 cm^2) of a ILM made of 30% v/v N,N-diethyl-dodecanamide in dodecane. The shell side of the separator was connected to the product vessel containing 500 mg of the enzyme Acylase I (EC 3.5.1.14) from Aspergillus Sp. (Aminoacylase AMANO, Nagoya, Japan), at pH 7.5.

The two phases were circulated at 25°C countercurrently, at the rates of 50 mL/min. (tube phase) and 500 mL/min. (shell phase), with the assistance of two peristaltic pumps using the configuration illustrated in Figure 2. Constancy of pH in both phases was secured through the use of pH stats.

The formation of N-formyl-(ß-methyl)-asp-phe was monitored by HPLC, using a Tracor Model 995 instrument together with a LDC Spectromonitor II detector set at 254 nm. The column used was a NOVA-PAK C18 Radial-Pak cartridge, 8 mm x 100 mm, housed in a Millipore Waters RCM-100 Radial Compression Module.

The mobile phases used for the analysis were:

(a) for N-formyl-(ß-methyl)-asp-phe-OMe: 40% v/v methanol in 0.1% KH_2PO_4 buffer pH 4.6;

(b) for N-formyl (ß-methyl)-asp-phe: 20% v/v methanol in 0.1% KH_2PO_4 pH 4.6; flow rates used were 1 mL/min for both analyses.

The data relating to the formation of N-formyl-(ß-methyl)- asp-phe (product dipeptide) is reproduced in Table V below, and is expressed as the total amount (mg) accumulated in the 200 mL shell phase as a function of time.

Table V

Time (min)	Amount peptide/product solution (mg)
60	130
120	230
180	280
240	360
300	400

A plot of the data of Table V is shown in Figure 5.

At the end of the run, HPLC analysis indicated the presence of 283 mg of N-formyl-(ß-methyl)-asp-phe-OMe in the tube phase. These values allowed to calculate the amount of peptide synthesized during the operation of the reactor.

P_{tr} = peptide transferred into shell phase = 400. $\frac{336}{322}$ = 417.4 mg;

P_o = initial peptide in tube phase = 433.8 mg;

P_T = peptide remaining in tube phase at the end of the run = 273 mg;

$P_{tr} = (P_o - P_T) + P_{syn}$;

$P_{syn} = P_{tr} - (P_o - P_T) = 266.6$ mg; and

$\bar{V}_{syn} = \frac{266.6}{5} = 53.5$ mg/hr. 100 mL.

The V_{syn} of 53.5 mg/hr. 100 mL coincides with the rate of synthesis (500 mg/hr. L) measured for the forward velocity in equilibration studies done with N-formyl-(ß-methyl)-L-aspartic acid and L-phenylalanine methyl ester in the presence of thermolysin.

The product solution (200 mL) was recovered, adjusted to pH 2 with 1 N HCl and extracted twice with 200 mL EtOAc. The combined extracts left a white residue upon evaporation, that after recrystallization from EtOAc/hexane yielded 100 mg of N-formyl-(ß-methyl)-L-asp-L-phe, $[\alpha]_D^{25°} = +0.70°$ (c, 0.29;MeOH), identical (IR, ^{13}C-NMR) to an authentic sample prepared by the batch hydrolysis of synthetic N-formyl-(ß-methyl)-L-asp-L-phe-OMe with Aspergillus esterase, $[\alpha]_D^{25°} = +0.80°$ (c, 0.29; MeOH).

Example 6.

The experiment described in Example 5 was scaled up in a Type 2 Hollow fiber Dialysis Membrane (Bend Research Inc.), containing 1 ft^2 of liquid membrane (30% v/v N,N-diethyl- dodecanamide in dodecane). The tube phase contained 40 g L-phe-OMe, 24 g N-formyl-(ß-methyl)-L-asp and 3.08 g thermolysin Daiwa (a total of 5×10^6 proteolytic units) in 400 ml water, adjusted to pH 7.0. After an incubation period of 1 hr at 40°C, the amount of 1,068 g (2.7 g/L) of N-formyl-(ß-methyl)-L-asp-L-phe-OMe was found to be present. The solution was cooled to 25°C, adjusted to pH 5.0 with 1 N HCl, and connected to the tube side of the hollow fiber separator. The shell phase was made of 400 mL water, pH 7.5, containing 2 g aminoacylase I (Amano). The two phases were circulated countercurrently at 25°C for 5 hrs., as described in Example 5. The results are summarized in Table VI and Figure 6.

Table VI

Time (min)	Amount peptide/product solution (mg)
30	204
60	251
120	445
180	634
240	748
300	843

At the end of the run, the amount of N-formyl-(ß-methyl)-asp-phe-OMe remaining in tube phase was 586 mg.

The highest transport value observed (404 mg/hr) during the first 30 min. is the result of the high initial peptide concentration produced during the preincubation period. Departure from equilibrium caused by the transport of peptide set in the synthesis of more peptide, thus establishing a steady state condition after the first hour into the run, at the expected level of 200 mg/hr (Figure 6).

Example 7.

An experiment similar to that of Example 5 was conducted, except for the use of 10.00 g of D,L-phenylalanine methyl ester instead of the L-enantiomer. The results, presented in Table VII and Figure 7, clearly show that the observed rate of formation of N-formyl-(ß-methyl)-L-asp-L-phe was one-half of that seen with L-phe-OMe (Example 5, Table V), as expected from the enantioselectivity of thermolysin and the prior results of Examples 1 and 2.

Table VII

Time (min)	Amount peptide/product solution (mg)
30	10.6
60	25.5
120	33.2
180	55.3
240	59.6
300	69.1

Example 8.

Membrane-assisted enzymatic resolution of D,L-phenylalanine methyl ester was utilized in accordance with the present invention as follows: To a solution of 1.0 g (5.6 mmoles) of D,L-phenylalanine methyl ester in

100 mL water, pH 7.5, was added 500 mg of aminoacylase I (Amano Pharmaceutical Co., Nagoya, Japan). The mixture was allowed to react at 25°C for 30 min., at the end of which the presence of 266 mg L-phe (1.6 mmoles) and 712 mg D,L-Phe-OMe (4 mmoles) was observed by HPLC analysis. This solution was fed to the tube (reaction) side of a hollow-fiber Celgard supported ILM separator ("Type 2 Hollow Fiber Selective Dialysis Membrane", Bend Research Inc.) containing 0.5 ft^2 of a 30% v/v N,N-diethyl-dodecanamide in dodecane liquid film in it. The product side of the membrane (shell side of the separator) was filled with 200 mL water adjusted to pH 2.0 with diluted HCl. The two phases were circulated through the separator countercurrently at the rate of 200 mL/min., with the assistance of peristaltic pumps (Figure 2). The separation proceeded through the continuous addition of D,L-Phe-OMe to the tube phase, done at a rate of about 1 g D,L-Phe-OMe per hour. A total of 30 mL of a 7% solution of D,L-Phe-OMe (2.1 g; 11.7 mmoles) in water pH 7.5 was added in a period of 2 hrs. The pH of both phases was kept constant by the use of pH stats.

The course of the resolution was followed by HPLC, on samples taken from both phases every 30 min. The HPLC instrumentation and procedures are those described in Example 5. The mobile phase used in this case was 20% v/v methanol in 0.1% KH_2PO_4 buffer pH 4.6; with a flow rate of 1 mL/min. The results, presented in Table VII and plotted in Figure 8, showed the accumulation of L-Phe in tube phase and of D-Phe-OMe in shell phase. At the end of the experiment both phases were recovered and worked out as follows:

(a) Tube phase: The contents (130 mL) were adjusted to pH 8.5 with 1N NaOH, then extracted with 2 x 50 mL EtOAc. The aqueous phase was then adjusted to pH 4.0 with 1N HCl and the solution passed through a 2.5 x 20 cm Dowex (RTM) 50 (NH_4) column. After washing

with 200 mL water, the product was eluted with 200 mL of 10% NH_4OH. The eluate was concentrated to 50 mL in vacuo, and the solution freeze-dried. Yield: 249 mg, white solid,
L-phenylalanine, $[\alpha]_D^{25°} = -29.2°$ (c, 2; H_2O), lit. (Aldrich) $[\alpha]_D^{25°} = -35.0°$ (c,2;H_2O), optical purity 92%.

(b) Shell phase: (200 mL) was adjusted to pH 8.5 with diluted NaOH and extracted with 2 x 50 mL EtOAc. The organic extract was dried over anh. Na_2SO_4, evaporated to dryness, dissolved in 50 mL water acidified to pH 3.0 with 1N HCl and then freeze-dried, to yield 989 mg (4.6 mmole) of D-Phe-OMe HCl, white solid, $[\alpha]_D^{25°} = -21.0°$ (c, 2; EtOH), lit. (Aldrich) $[\alpha]_D^{25°} = -32.4°$ (c, 2; EtOH), optical purity 83%.

Based on the permeability value of 32 mg/cm^2. min found for Phe-OMe (water, pH 8, 25°C) on this membrane, the expected flux for a membrane area of 450 cm^2 (0.5ft^2) was 880 mg/hr. Table VIII shows that the amount of Phe-OMe transferred into the shell phase at the end of the first hour was 860 mg, suggesting that the transport was operating under membrane-limiting conditions.

Table VIII

D,L-Phe-OMe	Tube Phase		Shell Phase
added (g)	L-Phe (mg)	D,L-Phe-OMe (mg)	D-Phe-OMe (mg)
1.0	266	712	0 (0 min)
1.7	473	617	537 (30 min)
2.4	615	531	860 (60 min)
3.1	743	628	1154 (90 min)

Batch resolution of D,L-Phe-OMe through the enantioselective hydrolysis of the methyl ester function

catalyzed by subtilisin A (a serine-type alkaline protease) has been recently disclosed [Shui-Tein Chen, Kung-Tsung Wang and Chi-Huey Wong, J. Chem. Soc. Chem. Commun. 1986, 1514]. A hydrolase is required for membrane resolution of racemic carboxylic acid compounds. A hydrolase which is an esterase which is an esterase such as aminoacylase I, α-chymotrypsin and subtilisin A can be utilized to resolve a D,L-amino acid compound such as D,L-phenylalanine methyl ester. The Aminoacylase I is generally preferred for the demethylation of peptides over other esterolytic enzymes such as subtilisin A and α-chymotrypsin having strong proteolytic activities.

If the above described resolution of D,L-phenylalanine methyl ester is utilized to produce a peptide as described in the present invention, the L-phenylalanine produced is methylated by standard methylation means known in the art.

This example may be adaptable for resolution of other racemic carboxylic acids. For example, a racemic carboxylic acid ester compound in an aqueous reaction mixture including a hydrolyzing enzyme can be hydrolyzed to form a charged enantiomeric compound and an uncharged enantiomeric ester compound in the aqueous reaction mixture. The uncharged enantiomeric ester compound is then transported from the aqueous reaction mixture across an ion rejection membrane of the present invention including Type 1 or Type 2 Hollow Fiber Selective Dialysis Membranes from Bend Research, Inc. In one type of example such as Example 8, the racemic carboxylic acid ester compound is a D,L-amino acid ester compound; the charged enantiomeric compound is a L-amino acid compound and the uncharged enantiomeric ester compound is a D-amino acid ester compound. It will be appreciated that the selection of enzymes and reaction conditions is within the understanding and knowledge of persons skilled in the art of the present invention.

Continuous or batch processing means are provided by this Example in that the desired enantiomer of the reactant can be produced and added to the reaction mixture.

Example 9.

Synthesis of N-formyl-(ß-methyl)-L-asp-L-phe in accordance with the present invention was conducted utilizing immobilized aminoacylase I and ion-exchange resins for the removal of permeable products as follows: To a solution of 10.0 g (48 mmoles) L-phenylalanine methyl ester and 6.0 g (35 mmoles) of N-formyl-(ß-methyl)-L-aspartic acid in 100 L deionized water, adjusted to pH 7.0, was added 770 mg of thermolysin (Daiwa Chemical Company, Osaka, Japan) representing a total of 1.2×10^6 proteolytic units. The resulting solution was incubated for 1 hr. at 40°C, when HPLC analysis indicated the presence of 383 mg of N-formyl-(ß-methyl)-asp-phe-OMe. The solution was cooled to 25°C, the pH adjusted to 5.0, and the solution was placed in a 200 mL vessel 40 connected to the tube side 46 of a hollow fiber separator 44 ("Type 2 Hollow Fiber Selective Dialysis Membrane", Bend Research, Inc.) containing 900 cm^2 (1 ft^2) of a hydrophobic liquid membrane made of 30% N,N-diethyl- dodecanamide in dodecane. The shell side of the separator 48 was arranged as a closed circuit made of a series of connecting vessels illustrated in Figure 9. The solution returning to the separator 44 was adjusted to pH 4.0 in order to protonate the L-phe-OMe copermeating with the dipeptide N-formyl-(ß-methyl)-asp-phe-OMe. Circulation through a column of Dowex 50 (Na+) 50 removed the positively charged L-phe-OMe, leaving the uncharged dipeptide in solution. The effluent was adjusted to pH 7.0 and submitted to the action of the aminoacylase I, immobilized over DEAE-Sephadex (R-TM) 52 [T. Tosa, T. Mori and I. Chibata, Agr. Biol. Chem. 33, 1053 (1969)]. The resulting dipeptide

N-formyl-(ß-methyl)-asp-phe was negatively charged at that pH, and was subsequently captured by the Dowex 1 (Cl-) resin 54. The effluent was returned to the membrane separator prior adjustment to pH 4.0, thus closing the loop.

The tube 46 (100 mL) and shell 48 (500 mL) phases were circulated at 25 °C countercurrently through the membrane separator, at the rates of 50 mL/min. (tube phase) and 120 mL/min. (shell phase).

Periodic sampling of the shell phase was done on the effluent from the second enzyme (E_2), (Figure 9, sampling port 56, before entering the Dowex 1 (Cl-) column 54) and the samples monitored by HPLC following the procedures described in Example 5. As expected, at this point of the circuit (Figure 9) no L-phe that could result from the enzymatic hydrolysis of L-phe-OMe by E_2 (see Example 8) was detected; only a circulating steady-state level of N-formyl-(ß-methyl)-asp-phe (average concentration: 54 mg/L) was observed, reflecting the continuous transfer of the dipeptide N-formyl-(ß-methyl)-APM across the membrane and its subsequent hydrolysis by E_2. The efficient trapping of the charged dipeptide by the Dowex-1 resin is indicated by the low concentration of it observed at point A throughout the run. A similar parallel experiment performed in the absence of Dowex-1 peptide in the shell phase, as could be anticipated from the results discussed above. Again, no L-phe was found in the circulating shell phase. A comparison of both experiments is seen in Figure 10, and Table IX.

Table IX

mg N-formyl-(β-methyl)-asp-phe/shell phase

Time (min.)	Exp.1. Dowex 1 present	Exp.2.without Dowex 1.
30	31.6	30.2
60	--	37.8
120	26.0	71.8
180	35.4	83.1
240	45.6	--
300	51.2	--

In addition to separating phenylalanine lower alkyl ester copermeating the ion rejection membrane into the product mixture utilizing ion exchange resins as described in this Example, aspartic acid copermeating the ion rejection membrane into the product mixture can be separated utilizing such resins. The species or product that cannot back-diffuse across the membrane from the product mixture can be removed utilizing such ion exchange resins. Also, other separation methods known in the art including but not limited to electrophoresis, electrodialysis and membrane separations which are equivalents of ion exchange resin separations can be utilized in the present invention.

Immobilizing the condensing enzyme allows the enzymatic reaction in the tube phase to be conducted at an initial reaction mixture pH preferred for optimum efficiency of the enzymatic reaction considering the reactants, product(s) and enzyme including the desired equilibrium of the enzymatic reaction. Optionally, the initial reaction mixture pH in the tube phase can be readjusted to a second reaction mixture pH prior to contact with the membrane so that the second reaction mixture pH will maximize the membrane efficiency in transporting the uncharged product from the tube phasse across the membrane into the shell phase. Figure 9 does

not show adjusting the initial reaction mixture pH in the tube phase to a second reaction mixture pH.

Similarly, the esterase in the shell phase can be immobilized and the pH of the product mixture in the shell phase can be adjusted and readjusted as necessary to effect the most efficient processing. Example 8 and the above Example provide additional means for efficient continuous or batch processing utilizing the present invention. In continuous processing the desired enantiomer of reactants and any copermeating compounds can be returned to the tube phase or reaction mixture.

Industrial Applicability

The present invention can be utilized to produce aspartame as well as other peptides including peptides which are pharmacologically active.

While specific embodiments of the invention have been shown and described in detail to illustrate the application of the inventive principles, it will be understood that the invention may be embodied otherwise without departing from such principles.

* C L M S *

37

52-567.519

CLAIMS

1. A method for the enzymatic synthesis of peptides comprising the steps of:

enzymatically condensing first and second amino acid compounds in an aqueous initial reaction mixture to form an uncharged peptide;

transporting the uncharged peptide into an aqueous reaction mixture by osmosis across a membrane that will not transport substantial amounts of the amino acid compounds; and

converting the transported uncharged peptide to a form that cannot be retransported across the membrane to the initial reaction mixture.

2. A method as claimed in claim 1 further comprising the step of removing the peptide form that cannot be retransported across the membrane to the initial reaction mixture.

3. A method as claimed in claim 1 further comprising the step of separating the first and/or second amino acid compound copermeating the membrane with the uncharged compound into the aqueous second reaction mixture.

4. A method as claimed in claim 3 further comprising the step of returning the first amino acid compound copermeating the membrane with the uncharged compound to the aqueous initial reaction mixture.

5. A method as claimed in claim 3 further comprising the step of returning the second amino acid compound copermeating the membrane with the uncharged compound to the aqueous initial reaction mixture.

* C L M S *

6. A method as claimed in claim 1, comprising the steps of:

condensing a N-acyl-β-substituted -L-aspartic acid having an α-carboxylate group with a phenylalanine lower alkyl ester having an α-ammonium group, in an aqueous reaction mixture including a condensation enzyme to form N-acyl-L-aspartyl-(β-substituted) -L-phenylalanine lower alkyl ester, an uncharged peptide;

transporting the uncharged peptide from the aqueous reaction mixture across an ion rejection membrane into a product mixture by osmosis;

and converting the uncharged peptide in the product mixture to a species that cannot back-diffuse across the membrane.

7. A method as claimed in claim 6 further comprising the step of removing the species that cannot back-diffuse across the membrane from the product mixture.

8. A method as claimed in claim 6 or claim 7 where the condensation enzyme is a peptide forming protease active in a pH range of 4.0 to 9.0.

9. A method as claimed in claim 8 wherein the condensation enzyme is a serine proteinase, thiol proteinase, carboxyl proteinase or metalloproteinase.

10. A method as claimed in claim 8 wherein the condensation enzyme is chymotrypsin, trypsin, substilisin BNP', Achromobacter protease, papain, pepsin, thermolysin or prolisin and the pH of the reaction mixture is from 4.0 to 9.0.

11. A method as claimed in claim 10 wherein the condensation enzyme is thermolysin and the pH of the reaction mixture is about 5.5.

12. A method as claimed in claim 9 wherein the pH of the aqueous reaction mixture is from 4.0 to 6.0.

13. A method as claimed in any of claims 6 to 12 wherein the uncharged peptide is converted to a charged species by cleavage with an esterase enzyme.

14. A method as claimed in claim 13 wherein the esterase enzyme is active in a pH range of 6.0 to 9.0.

15. A method as claimed in claim 13 wherein the esterase enzyme is aminoacylase I, α-chymotrypsin or substilisin A.

16. A method as claimed in claim 15 wherein the esterase enzyme is aminoacylase I and the pH of the reaction mixture is about 7.0.

17. A method as claimed in any preceding claim wherein the membrane comprises a water-immiscible organic liquid immobilized by capillarity in pores in a microporous sheet, the organic liquid being a solvent for the uncharged peptide.

18. A method as claimed in claim 17 wherein the microporous sheet is made of polytetrafluoroethylene or polypropylene and the solvent comprises n-1-decanol, n-hexadecanol, n-dodecanol N,N-diethyl-dodecanamide, dodecane, 2-undecanone or mixtures thereof.

19. A method as claimed in claim 18 wherein the microporous sheet comprises polypropylene hollow fibers and the solvent comprises N,N-diethyl-dodecanamide and dodecane.

20. A method as claimed in claim 17 wherein the membrane is a Bend, Type 2 Hollow Fiber Selective

Dialysis Membrane.

21. A method as claimed in any preceding claim wherein the first amino acid compound is D,L-phenylalanine methyl ester, and the second amino acid compound is N-acyl-β-substituted -L-aspartic acid having an α-carboxylate group.

22. A method as claimed in claim 21 wherein the N-acyl group is ϕCH_2OCO-, $-CH=O$ or ϕCH_2CO- and the β-substitutent is ϕCH_2O-, ter BuO-, CH_3O- or NH_2-.

23. A method as claimed in any preceding claim wherein the condensation enzyme is immobilized.

24. A method as claimed in claim 23 wherein the condensation enzyme is thermolysin and the pH of the reaction mixture is about 7.0.

25. A method as claimed in claim 23 further comprising the step of readjusting the pH of the reaction mixture to a pH of about 5.0 prior to transporting the uncharged peptide from the reaction mixture across the ion rejection membrane.

26. A method as claimed in claim 6 further comprising the step of separating N-acyl-β-substituted-L-aspartic acid copermeating the ion rejection membrane into the product mixture.

27. A method as claimed in claim 6 further comprising the step of separating phenylalanine lower alkyl ester copermeating the ion rejection membrane into the product mixture.

28. A method for the enzymatic resolution of racemic carboxylic acid compounds, comprising the steps of:

hydrolysing a racemic carboxylic acid ester compound in an aqueous reaction mixture including a hydrolyzing enzyme to form a charged enantiomeric compound and an uncharged enantiomeric ester compound in the aqueous reaction mixture; and

transporting the uncharged enantiomeric ester compound from the aqueous reaction mixture across an ion rejection membrane into a product mixture.

29. A method as claimed in claim 28 wherein:

the racemic carboxylic acid ester compound is a D,L-amino acid ester compound;

the charged enantiomeric compound is a L-amino acid compound; and

the uncharged enantiomeric compound is a D-amino acid ester compound.

30. A method as claimed in claim 29 further comprising the step of converting the uncharged D-amino acid ester compound in the product mixture to a species that cannot back-diffuse across the membrane.

31. A method as claimed in claim 30 wherein the step of converting is adjusting the pH of the product mixture to a pH of about 2.0.

32. A method as claimed in claim 28 wherein the hydrolyzing enzyme is selected from the group consisting of aminoacylase I, α-chymotrypsin and subtilisin A.

33. A method as claimed in claim 28 wherein the hydrolyzing enzyme is immobilized.

34. A method as claimed in any of claims 28 to 33 wherein the membrane comprises a water-immiscible organic liquid immobilized by capillarity in pores in a microporous sheet, the organic liquid being a solvent

for the uncharged compound.

35. A method as claimed in claim 34 wherein the microporous sheet is made of polytetrafluoroethylene polypropylene and the solvent comprises n-1-decanol, n-hexadecanol, n-dodecanol, N,N-diethyl-dodecanamide, dodecane, 2-undecanone or mixtures thereof.

36. A method as claimed in claim 35 wherein the microporous sheet comprises polypropylene hollow fibers and the solvent comprises N,N-diethyl-dodecanamide and dodecane.

37. A method as claimed in claim 34 wherein the membrane is a Bend, Type 2 Hollow Fiber Selective Dialysis Membrane.

38. A method as claimed in claim 29 wherein the D,L-amino acid ester compound is D,L-phenylalanine lower alkyl ester having an α-ammonium group.

39. A method as claimed in claim 38 wherein the D,L-phenylalanine lower alkyl ester is D,L-phenylalanine methyl ester.

40. A method as claimed in claim 1 wherein said first amino acid compound is a charged L-amino acid compound produced by a method as claimed in any of claims 28 to 39.