COVALENT BINDING OF METHIONINE AND
TRYPTOPHAN TO SOY PROTEIN

by

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ABSTRACT

One common method of improving the nutritional quality of certain food proteins is through fortification with necessary amounts of limiting essential amino acids. This simple and convenient method, however, is not the best. Several disadvantages are associated with the addition of free amino acids to food proteins, such as changes in flavor and color, losses of added amino acids during food processing or cooking, differences in stability and metabolism between free amino acids and amino acids in proteins. Covalent attachment of the limiting amino acids, however, should eliminate these problems, and moreover, could improve the nutritional and functional properties of food proteins. In this study, therefore, an attempt to improve the nutritional value of the soy protein was made by using the carbodiimide condensation reaction to covalently bind methionine and tryptophan to soy protein. In order to confine as much as possible the binding of amino acid to the protein α -carboxyl groups the soy protein isolate (SPI) was partially hydrolyzed with pepsin to increase the number of α -carboxyls in soy protein. Various conditions of the carbodiimide reaction were analysed by a fractional factorial design in an attempt to determine the factors affecting the amino acid binding to soy protein hydrolysate (SPH). Of the factors investigated, pH, SPH concentration, carbodiimide concentration, activation time and reaction time were found to significantly affect the methionine binding efficiency, whereas pH, SPH concentration, carbodiimide concentration, amino acid concentration and reaction temperature were found to significantly influence the tryptophan binding efficiency to SPH. To determine the best level for each of the selected

factors an optimization of the carbodiimide reaction conditions was conducted by carrying out another factorial experiment. Thus, under the best condition found, the methionine and tryptophan contents of methionine - and tryptophan-bound SPH samples were increased 7.7-fold and 18.0-fold, respectively.

An in vitro pepsin-pancreatin digestion test demonstrated that the bound amino acids were readily released.

In order to improve the low yield of the final product, another analysis of the carbodiimide reaction conditions was carried out. Since the yield could not be markedly improved by this factorial design in which peptic SPH was used, SPI without preliminary hydrolysis was used as the starting material. A product with 95-99% yield was obtained and its methionine or tryptophan content was increased 6.3-fold or 11.3-fold, respectively. High digestibility was still maintained for these products.

Gel filtration chromatography demonstrated that the carbodiimide reaction caused an increase in the molecular weights of soy protein fractions. Furthermore, gel filtration chromatography revealed that, there was no selective amino acid binding among the different soy protein fractions during the carbodiimide reaction.

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INTRODUCTION

The efficient use of a protein by man or animal requires that it contains the essential amino acids as well as nitrogen in amounts and proportions needed by the organism to meet its needs for specific as well as for general physiological functions. It is well known, however, that with the possible exception of only a very few, most proteins do not contain the essential amino acids in the amounts and proportions needed by man or animal. Therefore, their utilization tends to be inefficient, particularly for protein foods derived from the vegetable kingdom (Bressani, 1975). The amino acid deficiencies of a protein source is usually overcome (balanced), as has been well documented, by the addition of the appropriate amounts of the deficient amino acids; these can be in the form of either crystalline (synthetic) compounds (protein fortification or complementation) or as constituents of proteins which are rich sources of the deficient amino acids (protein supplementation). Addition of synthetic amino acids results in increased protein quality due to the improvement of the essential amino acid pattern and due only to that, since there are no other parameters involved (Bressani, 1974). On the other hand, addition of the deficient amino acids as protein can make changes in all three major parameters affecting protein utilization, namely, protein quality, protein quantity, and the total energy value (Pellet, 1974).

Fortification with amino acids is the normal practice in formulating animal feeds (Waddell, 1958). Studies by Howe et al. (1965A, 1965B, 1967) have demonstrated the improvement in quality which can be obtained by fortifying foods with amino acids. Bread supplemented with

lysine is a far superior source of protein than ordinary bread (Altschul, 1974). It has been estimated, moreover, that 10,000-20,000 tons of l-lysine will by 1980, be added to cereals intended for human food (Chem. Engng News, 1973). The rapid proliferation of meat analogs from soy and other vegetable protein sources will increase the use of methionine in foods based on meat and milk model (Horan, 1974B). Bressani (1975), reported experimental results from human subjects indicating that addition of the limiting amino acids represents not only a better protein nutrition for the individual, but a significant economic saving as well, due to the increased efficiency with which protein is utilized. Although the interest in amino acid fortification of human foods is certainly on the increase, the utilization of this option, at the present time, is not sufficiently used to make a significant contribution to human protein supply. Fortification with amino acids, however, is expected to become an essential and regular adjunct of the human food system (Altschul, 1974). In this way, the technology will contribute more flexibility to counteract the pressures of greater populations and higher expectations.

The fortification of food proteins with essential amino acids is an important, simple and convenient method for improving their nutritional quality. However, it is not necessarily the best method of improving their nutritional quality. This is due to several defects associated with the addition of free amino acids to food proteins. Firstly, the added amino acids can easily be lost during the subsequent food processing, cooking or through washing or discarding of the juices (Bressani, 1975; Fujimaki et al., 1977). Secondly, it may easily occur

that the free amino acids suffer degradation or reaction with food ingredients (e.g. Strecker degradation or amino-carbonyl reaction) to affect the food qualities such as flavor and color (Yamashita et al., 1970). Thirdly, considering the methionine fortification of foods, its addition is severely restricted, because of the unattractive flavor which methionine, in general, imparts to foods (Kläui, 1974). Furthermore, Beigler (1969) reported that methionine gives off an unpleasant odor upon high heating, such as sterilization. This finding was recently confirmed by Hippe and Warthesen (1978) who reported that methionine fortification in products that receive high heat treatment is not feasible from a flavor standpoint because of the formation of methional and dimethyl disulfide. A generally accepted reaction scheme for the production of flavor compounds from methionine is Strecker degradation (Ballance, 1961). Methional, a product of this reaction, has been reported to have a flavor threshold in the ppb range (Patton and Josephson, 1957). Fourthly, a difference in stability between free methionine and methionine in peptides has been reported by Arai (1974), suggesting the existence of different stability characteristics between free amino acids and amino acids covalently bound to proteins. Fifthly, the metabolism of amino acids ingested as proteins differs somewhat from that of free amino acids. More rapid intestinal transport of amino acids from small peptides than from the equivalent amount of free amino acid or amino acid mixture has been shown to occur in man and several other mammalian species by many techniques including tolerance tests, the use of tied loops of intestine or intestinal perfusion (Craft et al., 1968; Matthews et al., 1968; Adibi, 1971),

and measurements of uptake or transmural transport by small intestine in vitro (Cheng et al., 1971; Rubins and Auricchio, 1971). Thus, differences have been reported in intestinal transport between oligopeptide methionine and free methionine (Lis et al., 1972) as well as between oligopeptide lysine and free lysine (Burston et al., 1972). One possible method for removing or lessening the above defects is to supplement the desirable amino acids in a form covalently bound with proteins or peptides in foods.

The high nutritive value of the soybean protein has been recognised for many years and has been responsible for the enormous growth of its use in the animal feed industry (Horan, 1974A). Insofar as the food industry is concerned, soy proteins until recently were selected or used primarily for their functional effects in imparting desirable properties to fabricated foods. Now, however, they are being incorporated for their nutritional value (Wolf, 1972A). Soy protein offers the only expanding commercial source of protein able to meet the growing demand for a nutritionally balanced, high protein food suitable for both human and animal feeding (Coppock, 1974). Soy protein is a good source of all the essential amino acids except methionine and tryptophan (Wolf, 1972A).

The specific objectives, therefore, of this work were: (1) Optimization of the carbodiimide condensation reaction used as a means for covalently binding methionine and tryptophan to soy protein; (2) evaluation of the nutritional quality of the chemically modified soy protein obtained under the best condition; and (3) determination of the effect, if any, of the carbodiimide condensation reaction on the molecular weight distribution pattern of the soy protein.

LITERATURE REVIEW

A. The chemistry of carbodiimides

In 1955 Sheehan and Hess introduced a new method for the formation of peptide bonds using N,N'-dialkylated carbodiimides, preferably N,N'-dicyclohexylcarbodiimide. This reagent became, in a short time, the most important coupling reagent. The carbodiimide method can be called the most popular method of peptide synthesis. The main reason for it has probably been the high reactivity of carbodiimides, since this assures that the desired coupling reactions proceed smoothly, usually to completion of the acylation within a short time (Bodanszky and Ondetti, 1966). In addition to this, the simplicity and convenience of this technique as well as the commercial availability of the reagent are also an explanation for this unprecedented popularity. Racemization is slight (Anderson and Callahan, 1958) to negligible (Rebek and Feitler, 1975) and this fact offers an additional advantage. The carbodiimide condensation reaction can be expressed in the following equation:

The reaction mechanism was first discussed by Khorana (1953, 1955) and was more recently thoroughly reinvestigated by Detar and his coworkers (1966A,B,C). Khorana (1953) suggested that the reaction proceeds through the activation of the carboxyl group (I), initiated by the addition of its proton to the double bond of the carbodiimide(II), followed by the attack of the carboxylate anion to form the unstable reactive intermediate(III), the O-acylisourea (Fig.1). This O-acylisourea(III), in turn, can undergo either an intramolecular rearrangement, via an oxygen to nitrogen (O→N) acyl transfer, to an N-acylurea(V) or conversion to two

Fig. 1. A mechanism proposed by Khorana (1955) for the carbodiimide-mediated peptide synthesis.

activated forms (IV and VI) of the original carboxyl component (e.g. carboxylic acids, peptides or proteins). In addition to the above mentioned possibilities, Franzblau et al. (1963) suggested that in certain cases, the O-acylisourea could cyclize. The first alternative, that is the formation of N-acylurea, has a chance to occur when there are no further protons available (Schröder and Lübke, 1965) or when no other nucleophile is present or the reaction of those present is slow or hindered (Kopple, 1966). If excess carboxyl component is the only available reactant a second addition of proton takes place and the O→N acyl migration will be prevented. Stabilization occurs by way of formation of the symmetrical anhydride (VI) and disubstituted urea (Va). Although the intramolecular rearrangement of O-acylisourea is probably the most common route to the formation of N-acylureas, an alternative mechanism for their production, namely, by the subsequent acylation of the initially formed disubstituted urea (Va) by the acid anhydride (VI), was discovered in experiments carried out by Smith et al. (1958). It has a chance to occur when carbodiimide and carboxyl component are kept for a long time at room temperature and no other reactants are present. This possibility, however, has been subsequently excluded by De Tar and Silverstein (1966A).

The synthesis of peptide derivatives (VII), according to Khorana (1955), may proceed through one or more of the following three routes:

(a) $(IV) + NH_2-R'' \rightarrow (VII)$, since O-acylisourea has been found to be sensitive to bases and reacts readily with nucleophiles (Khorana, 1952); (b) acid anhydride $(VI) + NH_2-R'' \rightarrow (VII)$; and (c) $N\text{-acylurea } (V) + NH_2-R'' \rightarrow (VII)$.

Support for the above mechanism has been provided by Doleschall and Lempert (1963), who isolated a cyclic O-acylisourea and examined its reactions with

different nucleophiles.

The N-acylureas of the type (V) appear to be quite stable and show little tendency to undergo condensation with an amino acid ester under mild conditions and thus conversion into desired peptide derivative (VII), (Khorana, 1953). It has been subsequently suggested, however, that N-acylureas are not activated compounds and therefore are unreactive toward amines and do not form peptide bonds. The N-acylurea (path c) has, thus, been excluded as an acylating agent (Diehl, 1957; De Tar and Silverstein, 1966A, Klausner and Bodansky, 1972).

Although the O-acylisourea is highly unstable and has not been isolated except in one very special case (Doleschall and Lempert, 1963), its occurrence is inferred from kinetic data and from the appearance in the reaction mixture of both the urea of the carbodiimide (as a by-product of peptide synthesis) and the N-acylurea resulting from the rearrangement of the proposed O-acylisourea (Steinman et al., 1966). Recently a mechanistic study of the carbodiimide-mediated peptide synthesis has been reported by Rebek and Feitler (1973), who have determined the reaction intermediates. Their evidence indicated that the initial acylating agent in the carbodiimide reaction was distinguishable from the carboxylic acid anhydride under conditions normally encountered during peptide synthesis in solution. Their results indicated a maximum of 55% (5°C) to 60% (30°C) as the extent of anhydride's participation during peptide synthesis in solution with N,N'-dicyclohexylcarbodiimide. Delayed addition of the amine component was found to decrease the importance of O-acylisourea (path a) and increase the extent of anhydride's participation (path b) in peptide synthesis.

One advantage of N,N'-dicyclohexylcarbodiimide claimed by Sheehan

and Hess (1955) was the apparent lack of racemization in the formation of optically active peptide derivatives. However, the fact that coupling reactions promoted by carbodiimides may sometimes be attended by appreciable racemization was indicated by the finding of Hofmann et al. (1958) and was promptly reaffirmed by Anderson and Callahan (1958), who have demonstrated an influence of solvent and temperature on the racemization. Low temperatures (-5°C) reduce significantly the amount of racemic mixture (DL-form). Recently Rebek and Feitler (1975), however, working on the peptide synthesis with carbodiimide, reported that racemization was in the range of 0.01 to 0.1%. From the racemization point of view, symmetrical anhydrides may be undesirable intermediates, since racemization might be expected from the intermediate anhydride on the basis of the ready azlactone formation (Bodanszky and Ondetti, 1966). Intermediary azlactone formation is the most likely course that racemization takes when occurring during a condensation reaction (Schröder and Lübke, 1965). With the widely used dicyclohexylcarbodiimide reagent, racemization increases with temperature and is more pronounced in polar solvents such as dimethylformamide than in nonpolar, e.g., CH_2Cl_2 (Bodanszky et al., 1976). The most efficient method to reduce racemization in coupling with carbodiimide is to use additives such as N-hydroxysuccinimide or 1-hydroxybenzotriazole. Unfortunately the use of additives cannot be taken as a guarantee against racemization (Bodanszky et al., 1976).

The undesired formation of N-acylurea is frequently observed (Khorana, 1955; Merrifield and Woolley, 1956; Shankman and Schvo, 1958; and Sheehan et al., 1956). It is dependent upon a number of factors. Low temperatures reduce its formation (Helferich and Böshagen, 1959; Schneider, 1960); therefore the reaction is normally carried out at a temperature of 0°C

or lower (du Vigneaud et al., 1957; Katsoyannis and Suzuki, 1962; Sheehan and Yang, 1958). However, in a few cases the reaction is carried out at higher temperatures (Theodoropoulos and Fölsch, 1958; Previero et al., 1973). The formation of N-acylurea is suppressed in methylene chloride (Merrifield, 1958; Merrifield and Woolley, 1958; Sheehan et al., 1956) or acetonitrile (Benoiton and Rydon, 1960; Sheehan et al., 1956). Khorana (1955) pointed out that the relative proportions of N-acylureas formed during the coupling reaction will depend largely upon the particular peptide derivative used and the solvent employed and that their formation even in relatively small amounts may complicate the isolation of the desired products in a satisfactory yield. Note was made too, that, since the rearrangement of O-acylisourea(III) to the N-acylurea (V) is intramolecular whereas the reactions(III) $\rightarrow$ (IV) $\rightarrow$ (VI) and (IV) $\rightarrow$ (VII) are bimolecular (intermolecular), the formation of the desired products should be favored by an increase in the concentration of the reactants. Tometsko (1973) illustrated that the activated carboxyl groups during peptide synthesis are not stable for long periods of time, since the formation of N-acylurea derivatives results in a loss of activated carboxyl component and therefore decrease in peptide bond formation. The inactivation is a complex process that could also involve solvent impurities, temperature effects, etc.

One problem associated with the use of the original carbodiimides, particularly N,N'-dicyclohexylcarbodiimide, was the purification of the desired products. On one hand, the N-acylureas have structures and solubility rather similar to those of the desired peptides and therefore it is sometimes difficult to remove N-acylureas in order to obtain a pure

product. On the other hand, as pointed out by Khorana (1955), the insoluble co-product (dicyclohexylurea) which is formed during the implication of dicyclohexylcarbodiimide as the condensing agent in peptide syntheses could sometimes prove undesirable as, for example, in cases involving the synthesis of the less soluble higher polypeptides, since the co-product and the peptide derivative may have similar solubility properties, thus complicating the isolation procedure. In order to overcome the purification problems caused by the formation of by-product ureas and N-acylureas, other carbodiimides with more favorable solubility properties have also been investigated (Sheehan and Hlavka, 1956; Sheehan et al., 1961). With these reagents, the by-product ureas and N-acylureas as well as any excess of reagent are easily removed by washing with dilute acid or water. Peptides have been synthesized in high yields and in very pure forms with these carbodiimides (Sheehan et al., 1961). The most generally useful of them have been found to be 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (hereafter abbreviated to EDC) and the corresponding hydrochloride salt (Sheehan et al., 1961). These and the other water-soluble carbodiimides (WSC) are commercially available and the reaction can still be carried out under mild conditions (Greenstein and Winitz, 1961). The reaction mechanism of these water-soluble carbodiimides has been assumed to be similar to that proposed in 1953 by Khorana (Hoare and Koshland, 1966). Although all water-soluble carbodiimides react similarly, the smaller reagents might be expected to have greater access to partially buried carboxyl groups. This expectation appeared correct in a recent study with tobacco mosaic virus protein (Means and Feeney, 1971), in which upto three additional carboxyl groups could be

modified with EDC which did not react with 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-p-toluenesulfonate (the other most widely used water-soluble carbodiimide). Sheehan et al., (1965) working with EDC demonstrated that the synthesis of pure oligopeptides was extremely rapid and facile. Although coupling was usually about 80% after 1 hr at room temperature, the time required for complete coupling varied from 24 to 66 hr. The use of EDC has continued to expand because of its suitability to peptide synthesis and its high stability.

The reaction between a water-soluble carbodiimide and the functional groups of a protein could be both numerous and complex. It is of obvious interest, therefore, to review the reactions of these unusual reagents with proteins. Sheehan and Hlavka (1957) published the first studies of the reaction between a protein and a water-soluble carbodiimide (in the absence of an added nucleophile), stating that the reaction produced cross-linking in gelatin. This cross-linking was presumably due to amide formation occurring between side-chain carboxyl and amino groups of the gelatin molecule. On the other hand, by carrying out the reaction between a protein and a water-soluble carbodiimide at pH values (pH 4.0), where coupling by nucleophilic groups (such as ϵ -amino group of lysine) on the protein should be totally inhibited, Franzblau et al. (1963) have noted that the reaction between gelatin and a water-soluble carbodiimide, in the presence of hydroxylamine as nucleophile, yielded hydroxamic acid derivatives of side-chain aspartic acid and glutamic acid residues. These workers also reported that α -carboxyl groups reacted more rapidly and quantitatively to produce hydroxamic acid derivatives than β - or γ -carboxyl groups which exhibited lower yields (about 50% and 40%, respectively). Of great interest is the work of Previero et al. (1973), who

reported that EDC could affect the carboxyl groups of an acidic peptide in different ways depending on their environment, namely, they demonstrated that the same reagent could be used for both selective activation of the C-terminal and blocking of the side-chain carboxyl groups in a single operation. These workers, thus, presented evidence which indicated that direct condensation without preincubation of an acidic peptide with EDC resulted in the coupling of two times as many amino acid ester moles as in the case of condensation after preincubation with EDC for 90 min. at 40°C. Their proposed explanation was that, when condensation took place after preincubation, the O-acylisourea initially formed isomerized to the inert N-acylurea derivative at the side-chain carboxyl group, while the C-terminal remained activated as an Oxazolinone (Azlactone) which could subsequently react with a suitable nucleophile. Direct condensation without preincubation, on the other hand, resulted in the participation of both C-terminal and side-chain carboxyl groups in the formation of peptide bonds. Riehm and Scheraga (1966), by studying the reaction between ribonuclease and a water-soluble carbodiimide (in the absence of nucleophiles), have investigated all the possible reactions between a protein and a water-soluble carbodiimide. They demonstrated that the reaction between ribonuclease and carbodiimide, at low pH (4.5) did not involve direct reaction between the reagent and amino or imidazole groups (nucleophilic groups) in the protein. This finding might be expected since at pH 4.5 most (if not all) of these groups would be protonated. On the other hand, the reaction between the protein and the water-soluble carbodiimide at high pH (9.5), followed by amino acid analyses, resulted in a product which contained fewer lysine residues

than did a ribonuclease hydrolysate. Therefore, it was concluded that the reaction between a protein and a water-soluble carbodiimide, at high pH, results in the guanidination of ϵ -amino groups, due to reaction between carbodiimide and ϵ -amino groups of lysine residues. The same authors also excluded any reaction between carbodiimide and side-chain hydroxyl groups of threonine, serine, and tyrosine residues. This finding is in accordance with the results of Sheehan et al. (1955, 1956), who reported that the hydroxyl function of threonine, serine, and hydroxyproline does not require masking prior to condensation. Hoare and Koshland (1966) have used a water-soluble carbodiimide for the selective modification of carboxyl groups in proteins in the presence of high concentration of nucleophiles. They have reported a procedure leading to rapid and quantitative modification of carboxyl groups under mild conditions. Horinishi et al. (1968) have also explored the reaction of water-soluble carbodiimides with proteins in the presence of nucleophiles. They reported that with undenatured proteins, partial reaction is commonly observed and seemed to be an indication of the degree of exposure of the carboxyl groups, since only the more accessible or reactive carboxyl groups reacted. In the presence of denaturants (e.g. guanidine), however, quantitative modification of protein carboxyl groups could be obtained.

B. Physical and chemical properties of soybean proteins

(a) Solubility

The majority of soy-proteins are classified as globulins. Such proteins are insoluble in water in the region of their isoelectric

points, but will dissolve in the isoelectric state when salts such as sodium or calcium chloride are added. If the pH is above or below the isoelectric point, a globulin will also dissolve in aqueous solutions in the absence of salts (Wolf and Cowan, 1975). When defatted meal is dispersed in distilled water at pH 6.5, nearly maximum protein solubility occurs. Raising the pH with alkali increases the solubility slightly, but adding acid reduces the solubility abruptly to a minimum in the range of pH 4-5, which is the isoelectric region. At lower pH values, the proteins become positively charged and redissolve (Wolf, 1972A). Solubility of the soybean globulins is also influenced by the co-existence of phytate in soybeans (Smith and Rackis, 1957). Another factor that affects solubility of soybean proteins is disulfide polymerization (Wolf, 1972B). Commercial soyprotein isolates vary in solubility because of processing variations (Nash and Wolf, 1967). Part of the insolubility of isolates arises during the isoelectric precipitation step (Nash et al., 1971). One reaction responsible for this insolubility appears to be the formation of protein polymers linked by disulfide bonds. When the isolates are treated with sulfhydryl compounds such as cysteine or mercaptoethanol, depolymerization occurs and partial solubility is regained. A portion of the isolates, however, remains insoluble even with added sulfhydryl compounds (Nash et al., 1971). Solubility of the proteins is often required in order to obtain desired functional properties, and because soluble ingredients are always easier to formulate into foods. Consequently, almost all soy protein concentrates and isolates are neutralized and sold as proteinate, with sodium proteinate the major form available.

(b) Molecular size distribution

Undenatured soy proteins are a complex mixture of molecules that differ in size, charge, and structure (Wolf, 1975). The distribution in molecular weight ranges from 8,000 to about 600,000 as demonstrated by ultracentrifugation (Wolf, 1970) or by gel filtration (Hasegawa et al. (1963). An ultracentrifuge separates the water-extractable proteins of defatted meal into four major fractions. These fractions are designated 2S, 7S, 11S, and 15S based on their sedimentation rates (coefficients). The 7S and 11S fractions comprise more than 60% of the total protein, and about 80% of the proteins have molecular weights of 100,000 and higher. The 2S fraction, representing about 20% of the total protein, contains the low molecular-weight proteins: several trypsin inhibitors, cytochrome C, allantoinase, and 2 globulins with unknown biological activity.

The 7S fraction makes up more than one-third of the total protein and contains at least four different types of proteins: beta-amylase, four hemagglutinins, two or more lipoxygenases, and a protein called 7S globulin (Wolf and Cowan, 1975). The 7S globulin is a glycoprotein (Koshiyama, 1969) and makes up over one-half of the total 7S fraction (Wolf and Sly, 1967). About one-third of the total protein is found in the 11S fraction. So far only one protein called the 11S globulin or glycinin has been found in this sedimenting fraction. The 11S protein has a molecular weight of about 350,000, which is typical of major globulins of other seeds (Wolf, 1972B). The 15S fraction makes up the remainder of the protein. This fraction has not been isolated and characterized, but based on its sedimentation rate appears to have a molecular weight of over half a million. Some workers have suggested

that this fraction is a polymer of the 11S protein (Wolf, 1975). The wide distribution of molecular sizes among the water-extractable soybean proteins is also observed by gel filtration. Seven protein fractions have been fractionated (Hasegawa et al., 1963) as compared to only four in the ultracentrifuge. Obara and Kimura (1967), however, working on the gel filtration fractionation of the water-extractable soybean proteins, separated only four protein fractions. Additional evidence for the complexity of the soybean proteins has been obtained by hydroxylapatite chromatography (Wolf and Sly, 1965), starch gel electrophoresis (Puski and Melnychyn, 1968; Shibasaki and Okubo, 1966), and immunoelectrophoresis (Catsimpooulas et al., 1968).

(c) Association-Dissociation reactions

A characteristic property of the 7S and 11S globulins is their ability to undergo rapid and reversible association-dissociation reactions under mild changes in ionic environment (Wolf, 1975). Thus, the 7S globulin at pH 7.6, 0.5 ionic strength exists as a monomer with a molecular weight of 180,000 to 200,000, but when the ionic strength is lowered to 0.1, the protein dimerizes to a 370,000 mol. wt. species (Koshiyama, 1968A). Likewise, the 11S globulin forms a faster-sedimenting form when ionic strength is changed from 0.5 to 0.1, but the extent of association is low (Naismith, 1955). When ionic strength is reduced furthermore, to 0.01, the 11S protein dissociates into smaller units that sediment as 7S and 2 to 3S entities (Wolf and Briggs, 1958).

(d) Subunit structure

The 7S and 11S globulins possess an additional property that adds to their complexity. Both proteins are built-up of smaller units (sub-

units) that interact very specifically to form the parent globulins, and there appear to be no sub-units common to the two proteins (Wolf, 1972A). The 7S globulin contains 9 amino-terminal residues, a fact that indicates that the molecule contains at least 9 polypeptide chains (Koshiyama, 1968B). The 7S structure is disrupted by the usual reagents capable of dissociating proteins into subunits. When the protein is dissolved in 8M urea or 4M guanidine hydrochloride, the mol. wt. decreases from 180,000 for the native form to 22,500-24,000 (Koshiyama, 1971). Dissociation into subunits also occurs on treatment with the anionic detergent sodium dodecyl sulfate (SDS). In SDS solution, the molecular weight of 7S globulin is 34,000 (Wolf, 1972A). Binding of about 40 molecules of detergent per subunit would account for the discrepancy in molecular weight under the different conditions of protein dissociation (detergent versus urea or guanidine hydrochloride). At high pH (0.01N NaOH) the 7S protein changes to a form with a sedimentation coefficient of only 0.4S (Koshiyama, 1968A). The 11S globulin has a quaternary structure consisting of 12 subunits. Isoelectric focusing of 11S protein in urea-mercaptoethanol solution, however, separates only 6 subunits. These results suggest that the 11S molecule (mol. wt. of 350,000) is actually a dimer of 2 identical monomers, each consisting of 6 subunits (Catsimpoilas, 1969). The quaternary structure of 11S molecule is disrupted under various conditions, including low ionic strength, high or low pH, high concentrations of urea, anionic detergents (e.g. SDS), and temperatures above 80°C (Wolf, 1972B). For reasons still unknown, the 11S globulin is more susceptible to irreversible changes in structure than the 7S globulin (Wolf, 1972A).

(e) Amino acid composition

The chemical composition of soy proteins is very important because of its relationship to nutrition and reactions of the proteins during processing. Soy proteins are a good source of all the essential amino acids except methionine and tryptophan (Wolf, 1972A). Methionine is the first limiting amino acid of soy proteins (Berry et al., 1962) and this deficiency needs to be considered when the proteins are used for nutritional purposes (Coppock, 1974). Largely on the basis of animal experiments, it has been generally assumed that the nutritive value of soybean protein products for human consumption would be enhanced by fortification with methionine (Liener, 1972). The available evidence indicates that meals of soybean products, flour, or protein concentrates are improved in protein quality upon fortification with methionine (Berry et al., 1962, 1966; Evans and McGinnis, 1946). Methionine has been added at 0.5% of soy protein in infant formulas and such formulas have been judged equivalent in protein value to the proteins of meat, egg, and milk (Fomon et al., 1973). Some studies also suggest that tryptophan or threonine is the second limiting amino acid of soy protein. The improvement in protein quality, however, brought about by adding methionine is so high that any other amino acid added causes only small increases (Bressani, 1975). Soybean proteins being high in lysine, the first limiting amino acid in cereals (Kato and Muramatsu, 1971), are useful as supplementing blends (Bookwalter et al., 1971; Horan, 1973). A mixture of soy protein concentrate and wheat flour is nutritionally superior to either protein source alone; thus, a soy: wheat protein ratio of 3:1 gives an optimum protein efficiency ratio (Wilding et al.,

1968). The various soybean protein fractions differ in overall amino acid contents. Insofar as amino acid composition is concerned, the most significant differences between the 7S globulins and the 11S globulins are the 5- to 6-fold higher contents of methionine, tryptophan, and $\frac{1}{2}$ -cystine in the latter (Wolf, 1972B).

MATERIALS AND METHODS

A. Materials

Soy protein isolate used in this study was a product of General Mills, Inc., Minneapolis, MN. Pepsin (Hog stomach mucosa 2X crystallized and lyophilized powder), pancreatin (porcine pancreas grade VI), L-tryptophan, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, were all products of Sigma Chemical Company. L-methionine and p-dimethylaminobenzaldehyde (practical) were obtained from Eastman Organic Chemicals. Sodium nitroprusside was purchased from Fisher scientific company. Pronase (B grade) was from Calbiochem, Los Angeles. 5-Sulfosalicylic acid (98%) was obtained from Aldrich Chemical Company, Inc.. Sodium dodecyl sulfate (SDS), specially pure 99%, was purchased from BDH Chemicals, Canada. The standard proteins, used in this study for molecular weight calibration, and their sources were: ribonuclease A (bovine pancreas, type I-A, 5X crystallized), α -chymotrypsin (bovine pancreas, 3X crystallized), serum albumin (bovine), and catalase (bovine liver) were all products of Sigma Chemical Company; myoglobin (sperm whale) was from Schwartz/Mann. The Sephadex G-200 gel (particle diameter 40-120 μ), and the Blue Dextran 2000, were products of Pharmacia Fine Chemicals, Upsala, Sweden.

B. Preparation of soy protein hydrolysate

12 g of soy protein isolate was dispersed in 500 ml of distilled water. The pH of the mixture was adjusted with HCl to 1.6. To this dispersion was added 120 mg of pepsin dissolved in 100 ml of distilled water, and the mixture was incubated at 35°C for a specified time (5 or 16 hr). The hydrolysis was stopped by neutralizing (pH 7.0) the incubation mixture with NaOH. The resulting product was, then, centrifuged at 9,150 x g for 15 min., and the supernatant was freeze-dried to obtain a powdered soybean protein hydrolysate.

C. Statistical analysis (Selection of influential factors and determination of their best levels).

Various conditions of the carbodiimide condensation reaction were investigated by a 2-level fractional factorial experiment (Taguchi, 1957; Appendix), in an attempt to determine the factors affecting the amino acid binding to soy protein. To determine the best level for each of the selected influential factors a 3-level factorial experiment was used (Taguchi, 1957; Appendix). In order to improve the low yield of the final product obtained (i.e. amino acid-coupled soy protein hydrolysate), another 3-level factorial analysis of various conditions of the carbodiimide reaction was carried out, thus determining the factors influencing the final product's yield as well as their best levels. All statistical data obtained in the above mentioned factorial experiments were analysed by a Monroe Model 1880 programmable calculator.

D. Covalent binding of methionine and tryptophan to soy protein hydrolysate

A specified amount of soy protein hydrolysate was dissolved in 15 ml of distilled water at room temperature. The pH of the solution was

adjusted to the appropriate level (set by the experiment). A specified amount of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride dissolved in 2 ml of distilled water was, then, added to the soy protein hydrolysate solution. Absolute ethanol (1.1 ml) was also added, if required by the experiment. After the specified time for the activation^a of the protein carboxyl groups, a specified amount of L-methionine or L-tryptophan in solution^b was added to the reaction mixture. The pH of the mixture was checked and adjusted to the level set by the experiment. The reaction was allowed to proceed at a specified temperature for a specified time. Then, 1.8 ml of glacial acetic acid was added to the mixture to stop the reaction (by destroying the excess EDC). Subsequently, the sample was dialysed in the cold (5°C) against 2 changes of 1M acetic acid for 1½ day, against running tap water for ½ day, and finally against one change of distilled deionized water for ½ day. Then, the sample was neutralized (pH 7.5), stirred for 10 min (on a magnetic stirrer), and freeze-dried.

E. Methionine determination

The methionine content of various samples was determined by the sodium nitroprusside colorimetric method of Lunder (1974/1975), with the following modifications: (a) 0.5 g sample was mixed with 2.5 ml HCl 20% into a hydrolysis tube. The tube was sealed with vacuum grease, carefully closed with a metallic clamp, and then placed in an air oven at 120°C for 10 hours; (b) 450 mg of activated charcoal (12 x 30 mesh, Witco Chemical, N.Y.) were used to discolourize the protein hydrolysate; and (c) 10 ml of 25% ethyl alcohol was used to wash the activated charcoal and elute the methionine adsorbed on it. The remainder of the procedure

^a If no activation was required by the experiment (i.e., activation time 0 min.), then, the addition of amino acid solution to the soyprotein hydrolysate solution preceded the carbodiimide addition.

^b The amino acid solution was prepared by adding drops of 1N HCl to a stirred dispersion of a specified amount of amino acid in 4 ml of distilled water and bringing to the volume (5 ml) by adding dist. water.

was carried out exactly as described by Lunder (1974/1975).

F. Tryptophan determination

The tryptophan content of various samples was determined by the spectrophotometric method of Spies (1967) after hydrolysis of samples with pronase (procedure W).

The p-dimethylaminobenzaldehyde (practical) was recrystallized twice from ethanol by adding distilled water to a warm (about 50°C) ethanolic solution of p-dimethylaminobenzaldehyde until crystallization was just initiated and then cooling the solution. A suspension of slightly greenish-white crystals was obtained. These crystals were harvested by suction filtration, air-dried, and kept in a desiccator away from light until used.

G. Nitrogen determination

The nitrogen content of soy protein isolate, soy protein hydrolysate, as well as methionine- or tryptophan-bound soy protein isolate and soy protein hydrolysate samples obtained under best reaction conditions was determined by the rapid micro-Kjeldahl procedure of Concon and Soltess (1973). The protein content of each sample was then calculated by multiplying the amount of its nitrogen content by a factor of 6.25.

H. Amino acid analysis

The samples were hydrolysed with p-toluenesulfonic acid according to the method of Liu and Chang (1971), at 110°C for 24 hr. Amino acids were analysed on a single-column system (Durrum Chem. Corp., Palo Alto, CA.) attached to a Phoenix Model M6800 Amino Acid Analyzer (Phoenix Precision Instrument Co.).

I. Enzymatic digestion test

The digestibility of various samples was determined by the original pepsin-pancreatin digest method of Akeson and Stahmann (1964) as modified by Stahmann and Woldegiorgis (1975), with the following change: to 5.0 ml of digestion mixture was added 5.0 ml of 14% sulfosalicylic acid. The flask was then shaken for 15-30 minutes (in a shaking water bath at 37°C) and removed from the shaker. The pH was adjusted to 2.2 by adding drops of 6N NaOH. The digest was then transferred to a 15 ml graduated tube and brought to volume by adding pH 2.2 citrate sample diluter. The sample was then centrifuged at 1,000 x g for 30 minutes, filtered through a dry Whatman No. 1 filter paper, then through a sintered glass UF filter directly into a sample tube, and stored in a freezer at -20°C or in a cold room (5°C) for amino acid analysis.

J. Gel filtration chromatography

(1) Preparation and operation

Gel filtration was carried out on Sephadex G-200 in a glass chromatographic column (Fisher and Porter) with an internal diameter of 2.0 cm and a length of 100 cm. The packed column was stabilized and equilibrated with 2 bed volumes of elution buffer (0.1M sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide, to prevent microbial growth, and 0.1% SDS) in an upward flow direction. The operating pressure was adjusted to give a flow rate of about 10.4 ml per hour during equilibration and subsequent runs. The column was operated in an upward flow direction. The inlet tubing was joined to a 3-way valve which was connected to a funnel (filled with elution buffer) and a 5 ml syringe. Sample containing 150 mg protein in 5.0 ml solution was applied to the column via the syringe. The valve

was then turned to connect the eluant to the column. The effluent was collected by a fraction collector (Gilson Medical Electronics, Wisconsin) set to take 5.2 ml fractions. The elution pattern of each protein sample was obtained by measuring the absorbance of each fraction in a 1-cm cell at 280 nm by a Beckman DB spectrophotometer, and plotting the value against the fraction's number directly on a recording chart by means of a Varicord recorder Model 43 (Photovolt Corporation, N.Y.) attached to the spectrophotometer.

The void volume (V_o) and the total bed volume (V_t) of the column were determined from the elution volumes of Blue Dextran 2000 and tryptophan, respectively, using absorbances at 280 nm. The column was calibrated with standards proteins.

(2) Solubilization of samples

The methionine- or tryptophan-bound soy protein hydrolysate or soy protein isolate samples with the highest coupling yields were insoluble in the phosphate buffer (eluant). Thus, different dissociation procedures were used to solubilize the amino acid-bound soy protein hydrolysate samples (which exhibited much greater solubility than the corresponding amino acid-bound soy protein isolate samples). The tryptophan-bound soy protein hydrolysate sample was solubilized as follows: in 5.0 ml of elution buffer were added 20 mg SDS and 1 drop of 2-mercaptoethanol. To this solution was dispersed 150 mg of tryptophan-bound soy protein hydrolysate and the mixture was stirred on a magnetic stirrer. Moderate heat was applied to facilitate the dissolution. The sample after being completely solubilized was filtered through a dry Whatman No. 1 filter paper (to remove any invisible micro-particle), and then applied to the column. Although, the soy protein

hydrolysate (control) was soluble in the elution buffer, the same solubilization procedure was applied to it in order to have a better control. Since the methionine-coupled soy protein hydrolysate sample exhibited slight solubility under the above described conditions, the following procedure was used for its solubilization: 150 mg of methionine-bound soy protein hydrolysate were added to 5.0 ml of distilled water. The pH of the dispersion was adjusted to 12.0 by NaOH and the mixture was stirred at room temperature for 30 min, then centrifuged at $3,090 \times g$ for 15 min and the supernatant was applied to the column. The same method was used to solubilize 150 mg of soy protein hydrolysate (control) and 150 mg of tryptophan-bound soy protein hydrolysate sample. However, by this solubilization procedure the tryptophan-bound SPH sample was not completely solubilized, and thus, only the supernatant was applied to the column.

RESULTS AND DISCUSSION

A. Preparation of soy protein hydrolysate

Soy protein isolate was used as the starting material in this study, in order to eliminate any interference during the carbodiimide-mediated coupling reaction due to the presence of non-protein components. Various linking modes are possible during the carbodiimide condensation reaction, i.e., linkings between the amino group of the amino acid being bound and C-terminal α -carboxyl or side chain aspartic β - or glutamic γ -carboxyl groups of the soy protein molecule or between the N-terminal α -amino or ϵ -amino groups of soy protein and the carboxyl group of the amino acid being bound. However, the nutritional availability of β - α and γ - α linkages

is uncertain and not well documented in the literature. Since it has been reported (Franzblau et al., 1963) that protein α -carboxyl groups react more readily and quantitatively with carbodiimides in the presence of nucleophiles to produce the corresponding protein derivatives than protein β - or γ -carboxyl groups, the SPI was partially hydrolysed with pepsin to increase the number of α -carboxyl and α -amino groups in soy protein so as to confine the amino acid binding mainly to the α - α peptide linkages.

B. Statistical analysis

Data concerning the reaction characteristics of each amino acid are of fundamental importance for providing optimum reaction conditions and thus obtaining high yields during peptide synthesis. In order to determine, therefore, the factors influencing the methionine and tryptophan binding to soy protein hydrolysate (SPH), various conditions of the carbodiimide condensation reaction were analysed by a 2-level fractional factorial design (Taguchi, 1957; Appendix). Table 1 shows the factors and their assigned levels investigated for their possible influence on amino acid binding to soy protein. Tables 2 and 3 show the analysis of variance of the 2-level fractional factorial design for methionine and tryptophan binding, respectively, to SPH. As it can be seen in Table 2, pH, SPH concentration, reaction time, carbodiimide concentration, activation time and pH x SPH concentration interaction were found to significantly affect the methionine binding efficiency. On the other hand, Table 3 shows that pH, reaction temperature, amino acid (tryptophan) concentration, SPH concentration, carbodiimide concentration, and pH x SPH concentration interaction significantly influenced the tryptophan binding to SPH.

Table 1 - Factors and their assigned levels investigated for their possible influence on amino acid binding to SPH.

Factor	Level	
	1	2
1. pH	5	8
2. Reaction temperature (°C)	22	40
3. Pepsin hydrolysis time (hr)	5	16
4. Amino acid concentration (mg)	80	140
5. SPH concentration (mg)	220	660
6. Carbodiimide concentration (mg)	80	140
7. Activation time (min)	0	60
8. Reaction time (hr)	1	24
9. Polarity (% EtOH)	0	5

Table 2 - Analysis of variance of the 2-level fractional factorial experiment for methionine binding to SPH.

Source of Variation	S.S.	D.F.	M.S.	F-value
pH (A)	1.93	1	1.93	64.33**
SPH concentration (E)	4.48	1	4.48	149.33**
Reaction time (F)	0.87	1	0.87	29.00**
Carbodiimine concentration (G)	1.00	1	1.00	33.33**
Activation time (H)	0.20	1	0.20	6.67*
A x E	0.35	1	0.35	11.67**
Error ^a	0.27	9	0.03	
Total	9.10	15		

^a The sum of square values for reaction temperature (B), pepsin hydrolysis time (C), amino acid concentration (D) and polarity (I) were very low and, therefore, incorporated into the error S.S.

* $F_{0.05}(1,9) = 5.12$.

** $F_{0.01}(1,9) = 10.60$.

Table 3 - Analysis of variance of the 2-level fractional factorial experiment for tryptophan binding to SPH.

Source of Variation	S.S.	D.F.	M.S.	F-value
pH (A)	9.30	1	9.30	35.77**
Reaction temperature (B)	2.10	1	2.10	8.08*
Amino acid concentration (D)	1.62	1	1.62	6.23*
SPH concentration (E)	7.02	1	7.02	27.00**
Reaction time (F)	1.00	1	1.00	3.85
Carbodiimide concentration (G)	3.24	1	3.24	12.46*
Activation time (H)	1.00	1	1.00	3.85
Polarity (I)	0.64	1	0.64	2.46
A x E	4.20	1	4.20	16.15**
Error ^a	1.54	6	0.26	
Total	31.66	15		

^a The sum of square value for pepsin hydrolysis time (C) was low and, therefore, incorporated into the error S.S.

* $F_{0.05}(1,6) = 5.99$.

** $F_{0.01}(1,6) = 13.70$.

In order to find the level (of the two assigned) for each of the determined influential factors, which more favourably affected the binding of each amino acid to SPH, the response curve for each factor should be constructed. Figs. 2,3,4,5 and 6 show the effect of pH, SPH concentration, carbodiimide concentration, activation time, and reaction time, respectively, on the rate of methionine binding to SPH, whereas Figs. 2,3,4,7 and 8 show the effect of pH, SPH concentration, carbodiimide concentration, tryptophan concentration, and reaction temperature, respectively, on the rate of tryptophan binding to SPH. As it can be seen in these figures, a pH 5 was found to be more favourable than pH 8 for the binding of both amino acids (Fig. 2). Moreover, a low SPH concentration (220 mg) and high carbodiimide concentration (140 mg) were found to favor the rates of binding of both amino acids (Figs. 3 and 4, respectively). Figs. 5 and 6 show respectively that, activation time of 0 min., namely, no activation (direct condensation), and long reaction time (24 hr) were more favourable for the binding of methionine to SPH than activation time of 60 min., and a short reaction time (1 hr). On the other hand, a high amino acid (tryptophan) concentration (140 mg) and a high reaction temperature (40°C) were found to favor the tryptophan binding to SPH (Figs. 7 and 8, respectively).

In order to determine the best level for each of the selected influential factors an optimization of the carbodiimide reaction was conducted by carrying out a 3-level factorial analysis of variance (Taguchi, 1957; Appendix). Table 4 shows the three (3) levels assigned for each factor affecting the binding of methionine and tryptophan to SPH. Footnotes a and b indicate the level for each of the non-influential factors

Fig. 2. Effect of pH on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH. The vertical lines represent the 95% confidence limits. The confidence limit was calculated as follows:

$$C.L. = \frac{\pm t(0.05) \text{ at D.F. error}}{\text{no. of data averaged}} \times \sqrt{\frac{S.S. \text{ error}}{D.F. \text{ error}}}$$

Fig. 3. Effect of SPH concentration on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH.

Fig. 4. Effect of EDC concentration on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH.

Fig. 5. Effect of activation time on the rate of methionine binding to SPH.

Fig. 6. Effect of reaction time on the rate of methionine binding to SPH.

Fig. 7. Effect of amino acid (tryptophan) concentration on the rate of tryptophan binding to SPH.

Fig. 8. Effect of reaction temperature on the rate of tryptophan binding to SPH.

which was kept constant in all experiments. The 3 levels assigned for each influencing factor were not chosen arbitrarily, but selected so as to approach the optimal level of the factor found by the 2-level fractional factorial design. Tables 5 and 6 show the analysis of variance of the 3-level factorial experiment for methionine and tryptophan binding, respectively. As it can be seen in Table 5, highly significant ($P < 0.01$) differences in methionine binding were brought about by the three assigned levels of pH, SPH concentration, carbodiimide concentration, and activation time, whereas no statistically significant ($P > 0.05$) differences in methionine binding were caused by the three levels of reaction time tested or by the interaction of pH x SPH concentration. Table 6 indicates that highly significant ($P < 0.01$) differences in tryptophan binding were produced by the tested three levels of carbodiimide concentration and reaction temperature, whereas no statistically significant ($P > 0.05$) differences in tryptophan binding were caused by the three levels of the other influential factors or by the pH x SPH concentration interaction.

In order to find the best level of each influencing factor among the assigned three levels the response curves of these factors should be constructed. Fig. 9 shows the effect of pH on the rate of methionine and tryptophan binding to SPH. It can be seen that, pH 6.5 was found to be the best among the three levels for the efficient binding of both amino acids. This factor (pH) has been mentioned in several papers using carbodiimides (Hoare and Koshland, 1966; Means and Feeney, 1971; Riehm and Scheraga, 1966), as having an optimum at 4.75, namely, a pH which should normally limit the reaction to the protein carboxyl groups,

Table 4 - Assigned levels^{a,b} for factors influencing the amino acid binding to SPH.

Factor	Amino Acid					
	<u>Methionine</u>			<u>Tryptophan</u>		
	<u>Level</u>			<u>Level</u>		
	1	2	3	1	2	3
1. pH	3.5	5.0	6.5	3.5	5.0	6.5
2. SPH concentration(mg)	160	220	400	160	220	400
3. Carbodiimide concentration(mg)	100	140	200	100	140	200
4. Amino acid concentration(mg)				100	140	200
5. Activation time(min)	0	20	40			
6. Reaction time (hr)	12	24	30			
7. Reaction temperature (°C)				30	40	50

^a Level for each of the non-influencing factors which was kept constant in all experiments of methionine-binding reaction to SPH: pepsin hydrolysis time (16 hr), amino acid concentration (120 mg), polarity (0% EtOH), and reaction temperature (22°C).

^b Level for each of the non-influencing factors which was kept constant in all experiments of tryptophan-binding to SPH: pepsin hydrolysis time (16 hr), polarity (0% EtOH), activation time (0 min), and reaction time (16 hr).

Table 5 - Analysis of variance of the 3-level factorial experiment
for methionine binding to SPH.

Source of Variation	SS.	DF.	MS.	F-value
pH(A)	19.29	2	9.65	16.16**
SPH concentration(B)	21.31	2	10.66	17.86**
Carbodiimide concentration(C)	9.37	2	4.69	7.86**
Activation time (D)	9.38	2	4.69	7.86**
Reaction activation time(E)	3.66	2	1.83	3.07
A x B	5.09	4	1.27	2.13
Error	7.16	12	0.60	
Total	75.26	26		

* $F_{0.05}(2,12) = 3.89$, $F_{0.05}(4,12) = 3.26$.

** $F_{0.01}(2,12) = 6.93$, $F_{0.01}(4,12) = 5.41$.

Table 6 - Analysis of variance of the 3-level factorial experiment for tryptophan binding to SPH.

Source of variation	SS.	DF.	MS.	F-value
pH(A)	1.97	2	0.99	1.30
SPH concentration(B)	4.06	2	2.03	2.67
Carbodiimide concentration(C)	71.04	2	35.52	46.74**
Amino acid concentration(D)	2.08	2	1.04	1.37
Reaction temperature(E)	44.21	2	22.11	29.09**
A x B	4.25	4	1.06	1.40
Error	9.12	12	0.76	
Total	136.73	26		

$$F_{0.05}(2,12) = 3.89, F_{0.05}(4,12) = 3.26.$$

$$F_{0.01}(2,12) = 6.93, F_{0.01}(4,12) = 5.41.$$

Fig. 9. Effect of pH on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH.

since at this pH most (if not all) of the protein nucleophilic groups (such as amino or imidazole groups) would be protonated (Riehm and Scheraga, 1966). On the other hand, Horinishi et al. (1968) reported that the WSC-promoted reaction between bacitracin A (a cyclic dodecapeptide having two carboxyl groups of aspartic and glutamic acids in the double ring structure) and glycine ethylester (nucleophile), as well as the reaction between lysozyme (egg white) and glycine ethylester, both had a broad flat optimum between pH 5.2 and 6.9. These workers also ascribed the drop of the degree of reaction at the acidic side to decomposition of WSC, and on the alkaline side to hydrolysis of glycine ethylester with alkali and to deprotonation from the tertiary amino group of WSC (i.e., to the drop of the reactivity of the reagents). Holbek (1976), furthermore, working on the EDC-mediated binding of lysine to wheat gluten, has noted that pHs 7.5 and 10.0 resulted in considerably greater coupling yields than pH 5.0, pH 7.5 found to be the best among these three levels tested. The finding that at pH 6.5 the amino acid coupling was higher than at pH 5.0 can be explained by taking into account the fact that the isoelectric point of soy protein falls in the range of pH 4.0-5.0. Therefore, with the precipitation of soy protein at pH about 5.0, there will be less accessibility for both the WSC to be bound to soy protein carboxyl groups to form the reactive intermediates (O-acylisoureas) and the amino acid to attack these and other reaction intermediates to form the desired protein derivatives (products). Moreover, it was recently suggested by Addy et al. (1973A) that the pH used in the carbodiimide-mediated synthesis of peptides in aqueous medium should leave the amino group of the amino acid (being bound)

unprotonated, although the carbodiimide and postulated O-acylisourea should be protonated. Therefore, the yield of peptide bond formation should rise to a maximum when this condition exists and fall quickly when the pH is low enough to protonate the amino group of the amino acid, thus preventing it from acting as nucleophile (since the unprotonated form of the α -amino group of the amino acid being bound is the real nucleophile). The pK values for the amino groups of methionine and tryptophan are 9.21 and 9.39, respectively, (West and Todd, 1961), that is, much higher than the recommended pH of 4.75. At pH 6.5, however, both conflicting requirements, i.e. low pH to limit the reaction to protein carboxyl groups and pH which should leave the amino group of the amino acid being bound unprotonated, are partly met. Since this pH (6.5) lies between the isoelectric point and the pK values of the amino groups of both amino acids, therefore, there will be a certain amount of unprotonated amino groups, although low, available for nucleophilic attack. On the other hand, at this pH the reaction between soy protein and EDC did not involve direct reaction between EDC and amino or imidazole groups in the protein, as will be demonstrated from the amino acid analysis below. Figures 10, 11 and 12 show, respectively, that low SPH concentration, high carbodiimide concentration, and high amino acid concentration (although not statistically significant) resulted in higher amino acid coupling yields than the other two assigned levels of these influencing factors. These findings are in accordance with the original note made by Khorana in 1955 which stated that an increase in the concentration of reactants should favor the formation of desired protein products over the formation of undesirable side-products (N-

Fig. 10. Effect of SPH concentration on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH.

Fig. 11. Effect of EDC concentration on the rate of methionine (solid line) and tryptophan (broken line) binding to SPH.

Fig. 12. Effect of amino acid (tryptophan) concentration on the rate of tryptophan binding to SPH.

acylurea derivatives). Horinishi et al. (1968) have demonstrated that the WSC-mediated coupling between carboxyl groups of acetylglycine or proteins and aliphatic amines at room temperature can be enhanced by increasing the amine or WSC concentrations. On the other hand, it is well known, that an excess of carboxyl component favors the formation of symmetrical anhydrides (Schröder and Lübke, 1965). Delayed addition of the nucleophiles, in particular, has been found to decrease the importance of O-acylisourea and increase the extent of anhydride's participation during peptide synthesis (Rebek and Feitler, 1973). Symmetrical anhydrides, however, have been claimed to be undesirable intermediates from the racemization point of view (Bodanszky and Ondetti, 1966).

Fig. 13 shows the effect of activation time on the rate of methionine binding to SPH. As it can be seen, activation time of 0 min., that is, direct condensation without pre-incubation of the SPH with EDC, resulted in higher methionine coupling than activation (condensation after pre-incubation of SPH with EDC) for 20 or 40 min. This finding may be explained by recalling the note of Previero et al. (1973), who reported that direct condensation without preincubation of an acidic peptide with EDC resulted in the coupling of two times as many amino acid ester moles per mole of peptide as in the case of condensation after pre-incubation of the same acidic peptide with EDC for 90 min. at 40°C. On the other hand, the extent of condensation of amino acid esters with non-acidic peptides was constant and did not change during the incubation with EDC. These workers suggested that peptides containing a carboxyl group only at the C-terminal position rapidly reach a degree of activation

Fig. 13. Effect of activation time on the rate of methionine binding to SPH.

which remains constant, while peptides containing a side-chain carboxyl group show an initial maximum of activation which subsequently decreases to about one half of its value. Taking into consideration the fact that acidic amino acids (glutamic and aspartic) represent about 33% of the total amino acid composition of soy protein, it might be expected that at least a few of the side-chain aspartic and glutamic carboxyl groups participated in the formation of peptide bonds in the cases at which direct condensation was used. These groups, however, did not contribute to coupling when condensation after pre-incubation was used. Another factor which may also explain the higher binding yields observed when no activation took place is the formation of the undesirable stable N-acylureas. As it has already been mentioned, N-acylureas are formed during the carbodiimide condensation reaction, when no nucleophiles are present in the reaction mixture or the reaction of those present is slow or hindered (Kopple, 1966). The formation of N-acylurea, therefore, should be expected to be much higher when condensation after pre-incubation is used, and moreover, it should be anticipated that the longer the pre-incubation time the higher the formation of N-acylurea would be. N-acylurea, however, is not an acylating agent and thus, its formation would lead to lower coupling yields.

Figure 14 shows the effect of reaction time on the rate of methionine binding to SPH. As it can be seen, the three assigned reaction time levels did not cause any significant difference in methionine binding to SPH. The decrease in methionine binding, however, corresponding to the 24 hr reaction time level could not be explained. Hoare and Koshland (1966) stated that the reaction between a WSC and a protein in the presence

Fig. 14. Effect of reaction time on the rate of methionine binding to SPH.

of nucleophiles is quite rapid. Moreover, Sheehan et al. (1965) working with EDC demonstrated that during the synthesis of oligopeptide derivatives coupling was extremely rapid (about 80% after 1 hr). However, the reaction time required for complete coupling varied from 24 to 66 hr. Therefore, it seems from Fig. 14 that the optimum reaction time (time required for complete coupling) varies not only with the nature of carbodiimide used (Greenstein and Winitz, 1961), but also with the carboxyl component involved (starting protein or peptide). Fig. 15 represents the effect of temperature on the rate (efficiency) of tryptophan binding to SPH. As indicated in this figure, a reaction temperature of 50°C resulted in a significantly higher tryptophan binding than a temperature of 40° or 30°C. This finding can be easily explained by taking into account the fact that the synthetic reaction of a peptide-bond formation from a peptide and a free amino acid is a strongly endergonic (endothermic) process (Yamashita et al., 1970). Therefore, when a temperature of 50°C is used during the WSC condensation reaction, there will be more energy available to the system to help overcome the energy barrier with concomitant higher yield of peptide bond formation. Holbek (1976) has also noted that a reaction temperature of 40°C resulted in higher lysine coupling to gluten than a temperature of 10° or 25°C. The carbodiimide-mediated peptide synthesis is usually carried out at room temperature, because it is easier to handle and also to reduce the formation of undesirable N-acylurea derivatives. However, since the formation of N-acylureas would be expected to result in lower tryptophan coupling yields (for N-acylureas are not acylating agents), the fact that the tryptophan binding was more efficient at 50°C than at 40° or 30°C might indicate

Fig. 15. Effect of reaction temperature on the rate of tryptophan binding to SPH.

that the N-acylureas formation at 50°C was perhaps not significant in this reaction. The data presented in the above mentioned response curves are summarized in Table 7, which shows the factors affecting the binding of each amino acid to SPH, and their best levels of those tested.

One problem encountered during this study was the low yield of the final product, namely, of the amino acid-bound SPH. Thus, in order to improve the final product's yield another 3-level factorial analysis of the carbodiimide reaction conditions was carried out. Table 8 shows the factors and their assigned levels analysed for their possible influence on the final product's yield. Footnote a of Table 8 indicates the reaction conditions (factors) which were held constant in all experiments of this factorial design. Tables 9 and 10, show the analysis of variance of this 3-level factorial experiment for methionine and tryptophan, respectively, used for selection of the factors influencing the final product's yield and optimization. As shown in Table 9, pH, SPH concentration, reaction temperature and reaction time significantly affected the methionine-bound SPH yields. On the other hand, Table 10 shows that only SPH concentration significantly influenced the tryptophan-bound SPH yields. Table 11 indicates the factors, which were found to significantly affect the yields of methionine- or tryptophan-bound SPHs, and their best levels. The highest final product's yield obtained (in this factorial design) was 36%, whereas the lowest one was 17%. The methionine and tryptophan contents of the samples with the highest yield (36%) were 2.3% and 4.3% (i.e., 2.5 times and 4.6 times as much as those of SPI), respectively.

Table 7 - Factors affecting amino acid binding and their best levels.

Factor	Amino acid	
	Methionine	Tryptophan
pH	6.5	6.5
SPH concentration(mg)	160	160
Carbodiimide concentration(mg)	200	200
Amino acid concentration(mg)	-	200
Activation time(min)	0	-
Reaction time(hr)	12	-
Reaction temperature(°C)	-	50°

Table 8 - Assigned levels^a for factors investigated by a 3-level factorial experiment for their possible influence on final product's yield.

Factor	Level		
	1	2	3
SPI concentration(%)	2.4	3.6	4.8
Pepsin concentration(mg)	50	100	150
pH	5	6	7
SPH concentration(mg)	200	350	500
Reaction temperature (°C)	25	35	50
Reaction time(hr)	6	12	16

^a Constant factors: pepsin hydrolysis time (16 hr), amino acid concentration (140 mg), carbodiimide concentration (140 mg), activation time (0 min).

Table 9 - Analysis of variance of the 3-level factorial experiment for selection of the factors influencing the methionine-bound SPH yields and optimization.

Source of Variation	SS.	DF.	MS.	F-value
SPI concentration(A)	8.06	2	4.03	0.94
Pepsin concentration(B)	2.32	2	1.16	0.27
pH (C)	268.90	2	134.45	31.49**
SPH concentration(D)	192.93	2	96.47	22.59**
Reaction temperature(E)	207.51	2	103.76	24.30**
Reaction time(F)	60.02	2	30.01	7.03*
A x B	97.85	4	24.46	5.73
A x C	27.45	4	6.86	1.60
Error	25.61	6	4.27	
Total	890.65	26		

* $F_{0.05}(2,6) = 5.14$, $F_{0.05}(4,6) = 4.53$.

** $F_{0.01}(2,6) = 10.43$, $F_{0.01}(4,6) = 9.15$.

Table 10 - Analysis of variance of the 3-level factorial experiment for selection of the factors influencing the tryptophan-bound SPH yields and optimization.

Source of Variation	SS.	DF.	MS.	F-value
SPI concentration(A)	4.25	2	2.13	0.33
Pepsin concentration(B)	17.89	2	8.95	1.37
pH (C)	29.05	2	14.53	2.23
SPH concentration(D)	71.92	2	35.96	5.52*
Reaction temperature(E)	59.72	2	29.86	4.58
Reaction time(F)	18.46	2	9.23	1.42
A x B	169.67	4	42.42	6.51
A x C	7.75	4	1.94	0.30
Error	39.11	6	6.52	
Total	417.82	26		

* $F_{0.05}(2,6) = 5.14$.

** $F_{0.01}(2,6) = 10.43$.

Table 11 - Factors affecting final product's yield and their best levels.

Factor	Amino Acid	
	Methionine	Tryptophan
pH	5	-
SPH concentration(mg)	500	500
Reaction time(hr)	16	-
Reaction temperature(°C)	50°	-

Since the yield of the final product could not be markedly improved by the above 3-level factorial design, in which peptic SPH was used as the starting material in the carbodiimide reaction, another attempt to further improve the yield was done by using SPI itself (i.e., without preliminary pepsin hydrolysis) as the starting material and the same reaction conditions under which the amino acid-bound SPH samples with the highest coupling yields were obtained. The yield so obtained was 95% and 99% (on a weight basis) for the methionine- and tryptophan-bound SPI samples, respectively. Moreover, the efficiency of methionine and tryptophan binding to SPI was still excellent, as will be demonstrated in the amino acid analysis section below.

C. Amino acid analysis

The amino acid composition of SPI, and methionine- and tryptophan-bound soy protein samples with the highest amino acid binding efficiency is reported in Table 12. These results show that the L-methionine content in the methionine-bound SPH sample was 7.22g/16gN, whereas its content in the SPI (control) was only 0.94g/16gN. Furthermore, it can be seen that the L-tryptophan content in the tryptophan-bound SPH sample was 17.05g/16gN, whereas its content in the SPI was only 0.95g/16gN. On the other hand, Table 12 shows that the L-methionine content in the methionine-bound SPI sample was 5.92g/16gN, and the L-tryptophan content in the tryptophan-bound SPI sample was 10.74g/16gN. The above results, therefore, very clearly demonstrate that the L-methionine and L-tryptophan added have been very efficiently bound to SPHs and SPIs. The fact that the L-tryptophan has been more efficiently bound in both cases (i.e., when SPH or SPI was used as the starting material) than the L-methionine may be attributed to the higher reactivity of L-tryptophan (due to the presence of the indole ring in its molecule),

Table 12 - Amino acid composition^a (g/16gN) of SPI and amino acid-bound soy protein samples with the highest coupling yields.^b

Amino Acid	Control SPI	Met-bound SPH	Trp-bound SPH	Met-bound SPI	Trp-bound SPI
Aspartic acid	11.41	10.57	8.82	10.84	10.32
Threonine	3.67	3.66	3.09	3.53	3.39
Serine	5.31	5.75	4.25	5.00	4.73
Glutamic acid	19.40	18.21	15.37	18.34	17.50
Proline	5.10	5.54	5.52	4.84	4.68
Glycine	3.76	3.72	3.26	3.52	3.32
Alanine	3.75	3.16	2.83	3.56	3.46
Valine	3.67	3.54	3.09	3.23	2.96
Methionine	0.94	7.22	0.94	5.92	0.83
Isoleucine	4.27	3.61	3.68	3.97	3.69
Leucine	7.20	6.20	5.22	7.13	6.61
Tyrosine	3.58	3.34	3.07	3.30	3.15
Phenylalanine	5.30	4.50	4.37	5.00	4.72
Lysine	5.83	4.45	5.17	5.71	5.25
Histidine	2.53	2.77	2.63	2.43	2.25
Tryptophan	0.95	0.94	17.05	0.90	10.74
Arginine	7.38	6.82	5.64	6.93	6.56
Cystine	0.92	1.00	0.99	0.87	0.84

^a Each value is the average of duplicate analyses.

^b Experimental conditions: (a) methionine binding: pH 6.5; SPH or SPI concentration, 160 mg; EDC concentration, 140 mg; activation time, 0 min; reaction time, 12 hr; pepsin hydrolysis time, 16 hr; methionine concentration, 120 mg; reaction temperature, 22°C; (b) tryptophan binding: pH 6.5; SPH or SPI concentration, 160 mg; EDC concentration, 200 mg; activation time, 0 min; tryptophan concentration, 140 mg; reaction temperature, 50°C; pepsin hydrolysis time, 16 hr; reaction time, 16 hr.

since it is known that the coupling reaction, in general, depends upon the reactivity of each amino acid (Tometsko, 1973). A selectivity in aqueous peptide synthesis mediated by EDC was also reported by Addy et al. (1973B), which was suggested to be due not only to chemical reactivity of the side-chains of the amino acids but also to size and geometry of their side-chains. However, the higher efficiency of tryptophan binding observed here is apparently due to the former reason (i.e., higher reactivity).

It is noteworthy that as indicated in Table 12, the methionine- and tryptophan-bound SPI samples possessed amino acid compositions (except for the bound amino acids) which were similar (or closely resembled) to that found for the SPI (control). Therefore, it may be concluded that the reaction between SPI and EDC in the presence of added amino acid (methionine or tryptophan), at pH 6.5, did not involve direct reaction between the EDC and amino or imidazole groups in the protein, since the reaction between the EDC and a nucleophilic group (amino or imidazole group) in the protein should yield amino acid derivatives which would be stable to acid hydrolysis (Riehm and Scheraga, 1966).

Although soybean protein contains about 33% acidic amino acids (aspartic and glutamic), the possible lesser involvement of side-chain carboxyl groups in the EDC-mediated reaction could be due to amidation of aspartic and glutamic acid residues in the soy protein in the forms of asparagine and glutamine residues. The amide groups of these residues, however, have been shown to be very resistant to the action of carbodiimides (Liberek, 1962; Bodanszky et al., 1976), since the formation of the obligatory active intermediate (O-acylisourea) with carbodiimides

requires that the carboxyl groups be free (Franzblau et al., 1963). Therefore, these groups would not participate in the EDC condensation reaction. Another factor which could also explain the lesser participation of side-chain carboxyl groups to EDC reaction is the finding of Franzblau et al. (1963), who reported that the unstable reactive intermediate O-acylisourea - in addition to generally accepted possibilities of formation of a stable N-acylurea, a symmetrical anhydride or a peptide derivative - could cyclize. Thus, in the case of an aspartic acid residue (in an α -peptide linkage) cyclization would yield a succinimide ring, and a glutamic acid residue (in α -peptide linkage) could form either a glutarimide or acylpyrrolidone ring. If present, succinimide structures, on subsequent acid hydrolysis, would give aspartic acid and glutarimide or pyrrolidone rings would yield glutamic acid. Therefore, the cyclized residues would again appear as unreacted amino acids. A third factor which could also partly explain the lesser involvement of side-chain carboxyl groups is the formation of undesirable N-acylurea as a by-product of EDC reaction, which (since N-acylurea is not an acylating agent) results in lower coupling yields. N-acylureas, however, have been suggested to be acid-labile (Riehm and Scheraga, 1966), thus, liberating the amino acid involved on acid hydrolysis. Undoubtedly a certain amount of N-acylurea can accompany the formation of desired peptide derivatives in the EDC reaction.

D. Enzymatic digestion test

It is well known that the nutritive value of a protein depends not only on the pattern of its amino acid composition, but also on the physiological availability of its amino acids. Of the several characteristics which

determine the nutritional quality of food proteins, their digestibility is of paramount importance. Although in the final analysis the nutritive quality of a protein must be evaluated by feeding trials, in vitro enzymatic methods for protein quality evaluation have been utilized for preliminary quality evaluations (Stahmann and Woldegiorgis, 1975). Even though, the enzymatic hydrolysis may not be complete, the amount of the limiting amino acid released by enzymatic digestion is a good indicator of the amino acid availability and hence protein quality. Table 13 reports the amount of each amino acid liberated by an in vitro pepsin followed by pancreatin digestion from the SPI (control) and the amino acid-bound soy protein samples with the highest binding yields (efficiencies). As it can be seen, 2.18g/16gN of L-methionine were released from the methionine-bound SPH sample as compared to only 0.25g/16gN of L-methionine released from the SPI, whereas 4.55g/16gN of L-tryptophan were released from the tryptophan-bound SPH sample as compared to only 0.47g/16gN of L-tryptophan released from the SPI. On the other hand, Table 13 shows that 1.56g/16gN of L-methionine was released from the methionine-bound SPI sample and 2.06g/16gN of L-tryptophan was released from the tryptophan-bound SPI sample. These results, therefore, clearly indicate that the bound methionine and tryptophan can be efficiently released from both the SPH and SPI treated samples by enzymatic digestion in vitro.

Table 13 - Liberated amino acids^a (g/16gN) from SPI and amino acid-bound soy protein samples with the highest coupling yields^b by pepsin-pancreatin digestion in vitro.

Amino Acid	Control SPI	Met-bound SPH	Trp-bound SPH	Met-bound SPI	Trp-bound SPI
Threonine	0.05	0.20	0.20	0.18	0.19
Valine	1.46	0.59	0.50	0.51	0.44
Methionine	0.25	<u>2.18</u>	0.09	<u>1.56</u>	0.13
Isoleucine	0.95	<u>0.73</u>	0.63	<u>0.75</u>	0.56
Leucine	2.74	1.58	1.34	2.62	2.19
Tyrosine	1.98	1.29	1.09	1.75	1.16
Phenylalanine	2.65	1.58	1.40	2.76	1.68
Lysine	1.51	1.24	1.09	1.38	0.97
Histidine	0.36	0.57	0.53	0.53	0.49
Tryptophan	0.47	0.54	<u>4.55</u>	0.49	<u>2.06</u>
Arginine	2.17	3.36	<u>2.28</u>	3.16	<u>2.64</u>

^a Each value is the average of duplicate analyses.
Nutritionally important amino acids only were shown.

^b For experimental conditions see footnote b on Table 12.

Gel filtration chromatography was used to study the effect, if any, of the carbodiimide condensation reaction on the molecular weight distribution pattern of soy protein. In order to determine the molecular weights of the different protein fractions the Sephadex G-200 column was calibrated with the standard proteins listed in Table 14. A linear standard curve was obtained by plotting the logarithm of the molecular weights of the standard proteins against the ratios of their elution volumes to the void volume of the column (Whitaker, 1963). Figure 16 shows the calibration curve of this column. Figure 17 represents the elution profile of SPH solubilized with SDS. As it can be seen, three protein fractions were separated, the molecular weights of which were estimated (from the calibration curve) to be 158,490, 70,000, and 4,924, respectively (in order of increasing elution volume). Figure 18 shows the elution pattern of tryptophan-bound SPH sample with the highest binding efficiency solubilized with SDS. This pattern was obtained after diluting (1:5 dilution) each collected fraction (5.2 ml) with eluting buffer. This dilution was necessary in order to obtain absorbance values being within the spectrophotometer's absorbance range (0.0-2.0). The first-eluted fraction of the Figure 18 appeared at the void volume of the column and thus its molecular weight could not be estimated. It is assumed to be very high, since the peak of catalase curve, whose molecular weight is 244,000, was collected in the fraction (tube) 35, whereas the peak of this protein fraction appeared at the tube 29, namely, was eluted 31.2 ml (6 tubes) before the catalase. The molecular weights of the two other protein fractions of Figure 18 were estimated to be 97,700 and 15,550. Fig. 19 represents a comparison of the elution profiles of SPH (Fig. 17, used as control) and tryptophan-bound SPH (Fig. 18). It can be clearly seen that the elution pattern from the peptic SPH shifted towards the high-molecular weight side as a

Table 14 - Proteins used in calibration of Sephadex G-200 column
eluted with 0.1M sodium phosphate buffer, pH 7.0,
and containing 0.1% SDS.

Proteins	V_e/V_o	Mol.Wt.	(Ref.)
1. Catalase	1.10	244,000	(102)
2. Bovine serum albumin (Monomer)	1.52	70,000	(112)
3. α -Chymotrypsin	2.03	22,500	(112)
4. Sperm whale myoglobin	2.14	17,500	(*)
5. Ribonuclease A	2.17	13,600	(112)
6. L-tryptophan	2.38	204	(*)

* Mol. Wt. provided by supplier.

Fig. 16. Calibration curve of Sephadex G-200.

Fig. 17. Gel filtration pattern of SPH solubilized with SDS on Sephadex G-200.

Fig. 18. Gel filtration pattern of tryptophan-bound SPH solubilized with SDS on Sephadex G-200. The elution pattern was measured after diluting (1:5) each fraction with the eluant.

Fig. 19. Gel filtration patterns of SPH (solid line) and tryptophan-bound SPH (broken line) on Sephadex G-200.

result of the EDC condensation reaction. Figure 20 shows the gel filtration pattern of SPH solubilized with NaOH (pH 12.0). Three fractions were, again, obtained, the molecular weights of which estimated from the calibration curve were 158,490, 70,000 and 6,300, respectively (in order of increasing elution volume). Figure 21 represents the elution pattern of the methionine-bound SPH sample with the highest binding efficiency, solubilized with NaOH (pH 12.0). As it can be seen four fractions were separated. The first-eluted fraction appeared at the void volume of the column, and thus, the estimation of its molecular weight was not possible. The other fractions were estimated to have molecular weights of 91,200, 28,900, and 13,600, respectively (in order of increasing elution volume). Figure 22 shows a comparison of the elution profiles of SPH (Fig. 20, used as control) and methionine-bound SPH sample (Fig. 21). It can be seen, again, that the EDC condensation reaction caused an increase in the molecular weights of soy protein fractions. This finding is in accordance with the results of several papers dealing with the use of carbodiimides in peptide synthesis. Sheehan and Hlavka (1957), have reported that the reaction of a commercial gelatin with a WSC (in the absence of nucleophiles) resulted in very rapid gelation attributed to interchain cross-linking by amide bond formation occurring between side-chain carboxyl and amino groups. Blout and Des Roches (1959), then, reported that through the use of carbodiimides as condensing agents the molecular weights of polypeptides could be increased significantly, and moreover, that in several instances they observed four-fold or greater increases in molecular weights. These workers also suggested that this increase in molecular weight occurred because of terminal inter-

Fig. 20. Gel filtration pattern of SPH solubilized with NaOH (at pH 12.0) on Sephadex G-200.

Fig. 21. Gel filtration pattern of methionine-bound SPH solubilized with NaOH (at pH 12.0) on Sephadex G-200.

Fig. 22. Gel filtration patterns of SPH (solid line) and methionine-bound SPH (broken line) on Sephadex G-200.

molecular peptide bond formation, but definite proof was difficult to obtain. However, indirect evidence that linear peptide bond formation was involved could be deduced from their observation that polypeptides with one terminal amide group (i.e., the carboxyl end of the polypeptide chain was blocked by amide formation) and one terminal amine group did not undergo molecular weight increases when treated with carbodiimides, and also from the results obtained with poly-L-proline. With this polypeptide no inter-molecular condensations other than terminal may occur and poly-L-proline did show a definite molecular weight increase when treated with carbodiimides. More recently, Mukerjee and Sri Ram (1965), by carrying out the reaction between bovine serum albumin (BSA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the absence of nucleophiles, and based on subsequent sedimentation studies, reported that some aggregation of the protein had occurred. These workers also reported that, on reaction of EDC with BSA in the presence of diglycine, part of the peptide apparently was conjugated to the protein.

In order to determine if there was any selectivity regarding the binding of added amino acid to different soybean protein fractions during the EDC reaction, the elution profiles of tryptophan-bound SPH sample, solubilized with NaOH (pH 12.0), at 280 nm^a (Fig. 23) and 210 nm^b (Fig. 24) were obtained, and consequently, the ratio of the three separated fractions (areas under the eluted peaks) for each elution profile was determined. The area ratio (%) of the three fractions of

^a Absorption maximum of proteins due to the presence of aromatic amino acids (tryptophan, tyrosine, and phenylalanine).

^b Absorption of the peptide bond (Pomeranz and Meloan, 1971).

Fig. 23. Gel filtration pattern ($A_{280\text{nm}}$) of tryptophan-bound SPH solubilized with NaOH (at pH 12.0) on Sephadex G-200. The elution pattern was measured after diluting (1:5) each fraction with the eluant.

Fig. 24. Gel filtration pattern ($A_{210\text{nm}}$) of tryptophan-bound SPH solubilized with NaOH (at pH 12.0) on Sephadex G-200. The elution pattern was obtained after diluting (1:20) each fraction with 0.1 M sodium phosphate buffer, pH 7.0.

Fig. 23 was (44.79):(36.46):(18.75), in order of descending molecular weight, as compared to the ratio of (45.91):(38.26):(15.83) of the corresponding fractions of Fig. 24. Since these two ratios are very close (the small differences between the corresponding areas % of Figs. 23 and 24 are negligible and can be attributed to the dilution effects), it is concluded that there was no selective amino acid binding among the different soy protein fractions during the EDC reaction.

GENERAL DISCUSSION

From the results of Table 12, it appeared that the L-methionine and L-tryptophan added could be very efficiently bound to both SPH and SPI by the EDC condensation reaction. Moreover, Table 13 indicated a high digestibility in both cases (i.e., amino acid bound SPH and SPI). The use of SPI (instead of SPH) as the starting material in this reaction would be more advantageous for the following reasons: (1) elimination of the pepsin hydrolysis step required for the production of SPH. Thus, the covalent binding procedure would be simplified to one-step process which would permit direct binding of amino acids to soy protein; (2) prevention of the loss of some amino acids which is associated with the pepsin hydrolysis step (see Table 12); and (3) very high product yields (95-99%) as compared to the low yields (maximum yield 36%) obtained when SPH is used as the starting material.

The results of Table 12 showed that the L-methionine content in the methionine-bound SPI sample was 5.92g/16gN; this value is 6.3 times the methionine level, 0.94g/16gN, of the unmodified SPI. On the other hand, the L-tryptophan content in the tryptophan-bound SPI sample (Table 12) was 10.74g/16gN, namely 11.3 times the tryptophan content, 0.95g/16gN, of the

SPI (control). Although such contents (5.92g/16gN methionine and 10.74g/16gN tryptophan) are considered to be a surplus for optimum amino acid nutrition, it is possible, however, to obtain a protein product with a satisfactory methionine or tryptophan content if the factors affecting the binding of each amino acid to soy protein (e.g., amino acid concentration, carbodiimide concentration, pH, etc.) are properly controlled upon the application of EDC reaction. A more practical way to decrease the content of these amino acids is to mix the products with SPI. A 12:20:1 mixture, for example, of methionine-bound SPI sample: SPI:tryptophan-bound SPI sample will contain 2.7g/16gN methionine, 0.9g/16gN cystine, and 1.1g/16gN tryptophan. Such a mixture will resemble the ideal protein proposed by the FAO/WHO Expert Committee (1973), in respect to the levels of sulfur-containing amino acids (3.5g/16gN) and tryptophan (1.0g/16gN). It is well known that, if not properly considered, the addition of an excess of the first limiting amino acid may result in a secondary deficiency and, thus, there will be no improvement in the overall quality of the diet (Bressani, 1975). Consequently, the final mixture should include both the first and second limiting amino acids in the amounts needed to maintain the proper ratio according to requirements of the reference pattern (FAO/WHO Expert Committee - suggested pattern).

A major obstacle in the application of chemical modification to food proteins is the potential health hazards. Although enzymatic procedures (Whitaker, 1977) offer advantages with regard to potential health hazards, chemical procedures are frequently more advantageous for different products due to a low cost (Feeney, 1977). The major advantage of EDC used in this study is the facile removal of excess reagent and the corresponding urea

by washing with dilute acid or water. Moreover, it has been reported (Aumuller, 1963) that the mammalian toxicity of carbodiimides is low (LD_{50} of dicyclohexylcarbodiimide was reported to be 2.6 g/kg in rats). However, the possibility that some EDC and urea derivatives were still remained in the final product, after its purification, could not be excluded. It is obvious, therefore, that a thorough biological evaluation is needed to assess the effect of chemical modification of soy proteins by EDC on their in vivo digestibility as well as any effect that modification might have on the toxicity of their derivatives (methionine- or tryptophan-bound soy proteins).

CONCLUSION

According to the data presented in this study, the carbodiimide condensation reaction appeared to be a very effective means for covalently binding methionine and tryptophan to soy protein and thus improving its nutritional quality. It is feasible to obtain a product with a specified methionine or tryptophan content by simply controlling the factors affecting the binding of each of these amino acids to soy protein during the carbodiimide reaction. It was found that the methionine and tryptophan added could be very efficiently bound to both soy protein hydrolysate and soyprotein isolate by the carbodiimide reaction. Moreover, a pepsin-pancreatin digestion test demonstrated a high digestibility in both cases. Soy protein isolate, however, was found to be more advantageous as starting material in the carbodiimide (EDC) reaction than soy protein hydrolysate, because of the simplification of the covalent binding procedure to only one-step process, prevention of the loss of some amino acids associated with the pepsin hydrolysis step, and above all, because of the very high product yields obtained in this case. The nutritional quality of the product obtained, as determined by amino acid composition, was found to be superior to that of soyprotein isolate (control).

Gel filtration chromatography elucidated the formation of higher-molecular weight substances from peptic hydrolysates of soy protein as a result of the EDC reaction. Gel filtration chromatography, moreover, revealed that there was not any selectivity concerning the binding of added amino acid to the different soyprotein fractions during the EDC reaction.

The carbodiimide reaction may be of special importance from the viewpoint of food processing in practice. Further research is required to determine the commercial and economic feasibility of this method as a means for improving the nutritional and functional properties of food proteins. Moreover, it should be emphasized that the safety and acceptability of the product as a human food must be carefully evaluated.

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APPENDIX

Fractional factorial experiment

(Taguchi, 1957)

A 2-level experiment for selection of the factors influencing the amino acid binding to SPH.

Fractional factorial design

Run No.	Factor level ^a														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1	1		1	1	1		1			1	1	1		
2	1	1		1	1	1		2			2	2	2		
3	1	1		2	2	2		1			1	2	2		
4	1	1		2	2	2		2			2	1	1		
5	1	2		1	1	2		1			2	1	1		
6	1	2		1	1	2		2			1	2	2		
7	1	2		2	2	1		1			2	2	2		
8	1	2		2	2	1		2			1	1	1		
9	2	1		1	2	1		1			2	1	2		
10	2	1		1	2	1		2			1	2	1		
11	2	1		2	1	2		1			2	2	1		
12	2	1		2	1	2		2			1	1	2		
13	2	2		1	2	2		1			1	1	2		
14	2	2		1	2	2		2			2	2	1		
15	2	2		2	1	1		1			1	2	1		
16	2	2		2	1	1		2			2	1	2		

^a See Table 1.

A scheme to detect the interactions

1. Assign the nine factors (to be tested for their possible influence on amino acid binding to SPH) to columns 1, 2, 4, 5, 6, 8, 11, 12, and 13:

Column 1	pH
2	reaction temperature
4	pepsin hydrolysis time
5	amino acid concentration
6	SPH concentration
8	reaction time
11	carbodiimide concentration
12	activation time
13	polarity

The columns shown on the connecting lines of the scheme are left blank for detection of interactions. The effect of interactions will appear in those columns, i.e.:

Column 3	pH x reaction temperature
7	pH x SPH concentration
9	pH x reaction time
10	reaction temperature x reaction time
14	carbodiimide concentration x amino acid concentration

The unused column(s) will be used for the calculation of the error term.

2. Assign 2 different levels to each factor.
3. Carry out runs 1 to 16 in random order.
4. Determine influential factors and interactions by carrying out the analysis of variance (see Tables 2 and 3).

Fractional factorial experiment

(Taguchi, 1957)

A 3-level experiment for optimization of amino acid binding to SPH.

Fractional factorial design

Run No.	Factor level ^a												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1				1				1	1		1	
2	1				2				2	2		2	
3	1				3				3	3		3	
4	1				1				2	2		3	
5	1				2				3	3		1	
6	1				3				1	1		2	
7	1				1				3	3		2	
8	1				2				1	1		3	
9	1				3				2	2		1	
10	2				1				2	3		2	
11	2				2				3	1		3	
12	2				3				1	2		1	
13	2				1				3	1		1	
14	2				2				1	2		2	
15	2				3				2	3		3	
16	2				1				1	2		3	
17	2				2				2	3		1	
18	2				3				3	1		2	
19	3				1				3	2		3	
20	3				2				1	3		1	
21	3				3				2	1		2	
22	3				1				1	3		2	
23	3				2				2	1		3	
24	3				3				3	2		1	
25	3				1				2	1		1	
26	3				2				3	2		2	
27	3				3				1	3		3	

^a See Table 4.

A scheme to detect the interactions.

1. Assign the five influencing factors, which are to be optimized, to columns 1,5,9,10 and 12:

(a) methionine binding

Column	1	pH
	5	SPH concentration
	9	carbodiimide concentration
	10	activation time
	12	reaction time

(b) tryptophan binding

Column	1	pH
	5	SPH
	9	carbodiimide concentration
	10	amino acid concentration
	12	reaction temperature

All of the unused columns will be used for the calculation of the error term.

2. Assign 3 different levels to each factor.
3. Carry out runs 1 to 27 in random order.
4. Carry out the analysis of variance (see Tables 5 and 6).
5. Construct response curves by plotting the average of the data calculated for each level of the factors.