Immunological Properties of Pepsin, Trypsin and/or Chymotrypsin Digests of Bovine α s₁-Casein

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Abstract Peptides were separated from α s₁-casein digested with pepsin, trypsin and/or chymotrypsin by ultrafiltration with a Diaflo membrane YM 5 (cut off: Mw. 5,000). Molecular weights of most peptides in the trypsin or chymotrypsin digest ranged from 3,460 to 245, while those of most peptides in the pepsin digest ranged from 1,400 to 181 and those in the digest with the combination of the 3 proteases were smaller than 1,400. The binding between intact α s₁-casein and its IgG antibody was little inhibited by the 3 protease digest, and inhibited slightly by the pepsin, trypsin or chymotrypsin digest; each digest required more than 1,000 times the concentration of intact α s₁-casein to give a 25% inhibition. The IgG antibody titer in antisera obtained via the 10th injection of the pepsin, trypsin or chymotrypsin digest was only one or two orders of magnitude lower than that in antiserum obtained by the injection of intact α s₁-casein, while the antibody titer induced by the 3 protease digest was four orders of magnitude lower. On the other hand, the anaphylactic sensitizing capacity of the antisera with regard to digests was more than three orders of magnitude lower than that of the antiserum with regard to intact α s₁-casein.

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Breast feeding is best for most babies but artificial feeds based on cow's milk must be available as alternatives or supplements. Although most babies thrive on bottle feeding with preparations based on cow's milk without any apparent ill effects, a few babies develop some form of sensitivity toward cow's milk and become unable to tolerate it because of gastrointestinal, dermatological or respiratory problems¹⁾. Hence, other alternative formulas have been developed for babies with proven or suspected cow's milk allergy.

A formula based on bovine whole casein digest with molecular weights of smaller than 1,000 as a source of amino acids has been shown to be satisfactory to some extent in lowering the sensitizing capacity of the casein²⁾. However, severe enzymatic digestion of the casein by a combination of several proteases leads to low palatability, bitterness, off-flavors and substantial loss of nutritional value³⁾. Digestion should be no more severe or prolonged than is required to lose antigenicity and immunogenicity.

We therefore, decided to investigate the immunological properties of punctual

casein digests, in terms of reactivity of digests with antibodies against parent casein, and the production of antibodies to digests and the anaphylactic-sensitizing capacity of the produced antibody. In a previous study⁴⁾, we prepared peptide fragments with molecular weights of smaller than 10,000 from bovine β -casein digested with TPCK-trypsin and/or V 8-protease, and showed that repeated injections of the digests evoked high levels of IgG antibody specific to intact β -casein. Of particular interest was our finding that peptide fragments with molecular weights of smaller than 10,000 possessed immunogenic activity which was comparable to that of intact β -casein.

In the present study, we prepared peptide fragments with molecular weights of smaller than 5,000 from bovine α s₁-casein digested with pepsin, trypsin and/or chymotrypsin. We investigated the capacity of the digests to evoke IgG antibody production and anaphylactic sensitization.

Materials and Methods

1. Materials

Bovine α s₁-casein was prepared from one Holstein cow's milk by the procedure described previously⁵⁾. Pepsin (2,420 units/mg) was obtained from Sigma Chemical Co. Ltd. Trypsin (9,000 units/mg) and chymotrypsin (10,000 units/mg) were purchased from Boehringer Mannheim GmbH.

2. Preparation of digests

1) Pepsin, trypsin and chymotrypsin digests

α s₁-Casein (1 g) was dissolved in 50 ml of 0.2 M acetate-HCl buffer, pH 1.0 (in the case of pepsin digest) or 0.02 M phosphate buffer, pH 8.0, (in the case of trypsin or chymotrypsin digest) containing 0.02% sodium azide. Protease (pepsin 10 mg, trypsin 5 mg or chymotrypsin 5 mg) was added to the α s₁-casein solution. After incubation at 37°C for 24 hrs, the pH value of each reaction mixture was adjusted to 4.6 and the precipitate was removed by centrifugation. After the pH value was readjusted to 7.2, the clarified supernatant liquid was subjected to ultrafiltration on an Amicon ultrafiltration cell Model 8200 with a Diaflo membrane YM 5 (cut off : Mw. 5,000) in order to obtain a peptide preparation with molecular weights of smaller than 5,000. Each permeate fraction was recovered and freeze-dried. About 54%, 21% and 82% of the substrate peptide was obtained from pepsin, trypsin and chymotrypsin digestions, respectively.

2) Pepsin, trypsin and chymotrypsin digest

As above, α s₁-casein (1 g) was digested with 10 mg of pepsin at 37°C for 24 hrs. After the pH value of the digest was adjusted to 8.0, 5 mg each of trypsin and chymotrypsin were added to the pepsin digest, and the mixture was further incubated at 37°C for 24 hrs. After incubation, the digest was subjected to ultrafiltration as described above, and the permeate was freeze-dried. About 92% of the substrate peptide was recovered from the combination of pepsin, trypsin and chymotrypsin digestions (hereafter referred to as the 3 protease digest).

3. Immunization

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Male rabbits, weighing about 2 kg, were immunized by intradermal injection with 5 mg of each digest and intact α S1-casein in 0.85% NaCl, emulsified with an equal volume of Freund's complete adjuvant (Nacalai tesque Inc.) at 7-day intervals for 70 days. Serum samples were collected every week and stored at -80°C before use.

4. Antibody measurements

Serum IgG antibody to intact α S1-casein and reactivity of the digests with IgG antibody to intact α S1-casein were determined by an enzyme-linked immunosorbent assay (ELISA) and its inhibition test, as described previously⁴⁾. A reaginic antibody was determined via a passive cutaneous anaphylaxis (PCA) test using female guinea pigs of the Hartly strain weighing about 250 g. The PCA antibody titer was expressed as the reciprocal of the highest dilution at which a positive reaction was induced⁶⁾.

5. Gel filtration

Gel filtration and estimation of molecular weight by gel filtration were carried out as described in a previous paper⁴⁾.

Results

1. Gel filtration of digests

The pepsin, trypsin, chymotrypsin and 3 protease digests were fractionated by gel

Fig. 1. Gel filtration of the digests on a Bio-Gel P-10 column.

A: pepsin digest, B: trypsin digest, C: chymotrypsin digest, D: 3 protease digest.

Arrows indicate elution positions of marker peptides; a: bovine pancreas trypsin inhibitor (Mw. 6,160), b: glucagon (Mw. 3,460), c: bacitracin (Mw. 1,400), d: L-Leu-Gly-Gly (Mw. 245), e: L-tyrosine (Mw. 181).

Fig. 2. Effect of the protease digest of α_{s1} -casein on binding between intact α_{s1} -casein and its IgG antibody.
 ▲ : pepsin digest, ■ : trypsin digest, □ : chymotrypsin digest, ● : 3 protease digest, ○ : intact α_{s1} -casein.

filtration on Bio-Gel P-10 (Fig. 1). Bovine pancreas trypsin inhibitor, glucagon, bacitracin, L-Leu-Gly-Gly and L-tyrosine were used as standard materials for the estimation of molecular weights of peptides in the digests. Major components in the trypsin and chymotrypsin digests were eluted at volumes between or equal to glucagon and L-Leu-Gly-Gly, whereas those in the pepsin digest were eluted at volumes between or equal to bacitracin and L-tyrosine. All components in the digest with the 3 protease combination were eluted after a volume equal to bacitracin.

2. Antigenic reactivity of the digests with anti-intact α_{s1} -casein antibody

The reactivity of the digests to anti-intact α_{s1} -casein antibody was examined via the ELISA inhibition test (Fig. 2). The binding between intact α_{s1} -casein and its IgG antibody was inhibited by the addition of pepsin, trypsin and chymotrypsin digests, although each digest required about 1,000 to 10,000 times the concentration of intact α_{s1} -casein to give a 25% inhibition. Little inhibition was observed via the 3 protease digest.

3. Antibody response to the digests

The IgG antibody responses to each digest and intact α_{s1} -casein in rabbits are shown in Fig. 3. A rapid and persistent response was induced via intact α_{s1} -casein. Antibody response was also induced via the digests. At the last stage of immunization, the antibody titer of antiserum to the pepsin, trypsin and chymotrypsin digests was only about one or two orders of magnitude lower than that of the antiserum to

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Fig. 3. IgG antibody responses in rabbits against intact α s₁-casein and the digests.

The serum antibody titer of the rabbit injected with intact α s₁-casein (○) or the digest (▲: pepsin digest, ■: trypsin digest, □: chymotrypsin digest, ●: 3 protease digest) was measured by ELISA. The titer shows the peroxidase activity: absorbance at 490 nm $\times$ dilution-fold of serum.

intact α s₁-casein, while the antibody titer of the antiserum to the 3 protease digest was about four orders of magnitude lower.

4. Anaphylactic-sensitizing capacity of the antisera to digests

A dilution series ranging from 10² to 10⁷ of the rabbit antiserum obtained after the 10th injection of intact α s₁-casein or each digest was prepared. Guinea pigs were passively sensitized by an intradermal injection of each diluted antiserum. Four hours after the antiserum injection, the guinea pigs were challenged with intact α s₁-casein solution containing Evans blue. After 30 min the animals were killed and the skin inverted in order to inspect the inside of the skin (Fig. 4). On the PCA test a positive reaction was induced not only by the antiserum to intact α s₁-casein but also by the antiserum to the pepsin, trypsin and chymotrypsin digests. However, the PCA titers were very low in the antisera with regard to the digests; i.e. 10⁶ of the antiserum to intact α s₁-casein, 10³ of the antiserum to trypsin and chymotrypsin digests and 10² of the antiserum to pepsin digestion. Moreover, even at a 10² dilution, the antiserum to the 3 protease digest did not induce a clear positive reaction.

Discussion

Cow's milk allergy can develop not only in formula-fed babies but also in breast-fed babies⁷⁾. Hence, alternative formulas have been developed for babies with a proven or suspected cow's milk allergy. One formula is based on soy-bean protein

Fig. 4. Typical profiles of PCA test of αs_1 -casein with antisera to the digests. Each antiserum was diluted 100-fold (1) and further serially 10-fold (2, 3, 4, 5 and 6). I: antiserum to intact αs_1 -casein, T: antiserum to trypsin digest, C: antiserum to chymotrypsin digest, P: antiserum to pepsin digest, M: antiserum to 3 protease digest.

isolates as a source of amino acids. However, adverse reactions to soy products have been reported increasingly over the past several years⁸⁾. One study found that 35 % of patients who had milk protein intolerance also had soy protein intolerance⁹⁾.

The effect of heat treatment on the antigenicity of cow's milk proteins has been investigated since the early years of this century¹⁰⁾. Recently, HEPPELL *et al.*¹¹⁾ proposed that heat denaturation of whey protein might be a logical and simple strategy for producing a hypoallergenic baby milk. However, heating can adversely affect nutritional quality: *i.e.* cause a loss of available lysine and the formation of new antigenic determinants^{1,6,12)}. Thus, this approach is not entirely satisfactory.

On the other hand, it is well known that the extended digestion of bovine caseins with different proteases leads to a digest devoid of sensitizing or eliciting capacity. A formula based on the digested casein has been developed, and in practice, has been shown to be safe in hypersensitive infants²⁾. However, this formula has some disadvantages such as low palatability, bitterness, off-flavors and loss of nutritive value, because of digests consisting of free amino acids and low molecular weight peptides such as di- and tri-peptides¹³⁾.

Although it is impossible to specify general and precise limits of molecular weights for peptide immunogenicity, generally speaking, peptides with molecular

weights smaller than 5,000 tend to be weakly immunogenic¹⁴⁾. In the present work, we investigated the antigenicity and immunogenicity of peptide fragments with molecular weights of smaller than 5,000 prepared from the punctual digests of α s₁-casein in order to obtain information about the relationship between the immunogenicity and molecular weight of bovine casein digests. We used pepsin, trypsin and chymotrypsin to prepare the casein digests, because these proteases are major endopeptidases in the gastrointestinal tract of infants. The digests drastically reduced antigenicity, in terms of reactivity with IgG antibody to intact α s₁-casein (Fig. 2). However, in terms of immunogenicity in rabbits determined by the production of IgG antibody to intact α s₁-casein, only the 3 protease digest led to a drastic reduction, and the pepsin, trypsin and chymotrypsin digests possessed the ability to produce the IgG antibody to a large extent (Fig. 3). These results indicate that digestion with the 3 protease combination (pepsin, trypsin and chymotrypsin) is effective for reducing both the antigenicity and immunogenicity of α s₁-casein, and pepsin, trypsin and chymotrypsin digestion still remains in the immunogenicity of the parent protein.

It is well known that the usefulness of *in vitro* techniques such as ELISA and the immunoprecipitation test for detection of allergic patients, is limited due to a poor correlation between the *in vitro* tests and provocation tests in patients¹⁵⁾. Consequently, *in vivo* assay PCA using guinea pigs was employed to determine the anaphylactic-sensitizing capacity of antisera induced by the digests. Even at 100-fold dilution of antiserum to the 3 protease digest, no clear PCA reaction was seen on the surface of the skin, although an anti-intact α s₁-casein antiserum diluted 10⁶-fold gave sensitization. The antiserum to the pepsin, trypsin and chymotrypsin digests induced a positive PCA reaction at 10²-fold, 10³-fold and 10³-fold dilution, respectively (Fig. 4). These facts suggest that, despite a high ability to produce IgG antibodies, the digests lose the ability to produce an anaphylactic-sensitizing antibody.

The 3 protease digest has been shown to possess molecular weights of smaller than 1,400. The pepsin digest contained some peptides larger than 1,400, although the molecular weights of most peptides were smaller than 1,400. Moreover, the trypsin and chymotrypsin digests contained most peptides with molecular weights ranging from 3,460 to 245 and some peptides larger than 3,460. Hence, this suggests that the limitation of molecular weight in order to cause the immunogenicity of α s₁-casein ranges from 5,000 to 1,400. Further limitation of molecular weight to cause antibody production of casein is now in progress.

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ペプシン、トリプシンおよびキモトリプシン消化牛乳 α S1-カゼインの免疫学的性質

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ペプシン、トリプシンまたはキモトリプシン単独、およびそれら3種の酵素すべてで消化した α S1-カゼインを限外濾過にかけ、分子量5,000以下のペプチドを分離し、それらの抗原性と免疫原性を調べた。酵素免疫測定法での未処理 α S1-カゼインとそのIgG抗体との反応は、3種の酵素すべてによる消化ペプチドではほとんど阻害を受けなかった。また、各単独酵素消化ペプチドにおいても、その25%阻害に要する量は、いずれも未処理 α S1-カゼイン量の1000倍以上であり、元の抗原性は顕著に低下していた。一方、それら消化ペプチドを家

兎に注射した場合、生産されるIgGクラスの特異抗体量、並びにそれら抗体のモルモットに対する受身皮膚アナフィラキシー誘導能は、3種の酵素すべてによる消化ペプチドにおいて最も低く、次いでペプシン単独、キモトリプシンとトリプシン各単独ペプチドの順に高くなった。これらの結果、およびそれら各消化ペプチドの分子量の測定結果より、牛乳 α S1-カゼイン消化物の免疫原性に関係する分子量域は1,400から5,000の範囲内にあることが示唆された。

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