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DEVELOPMENT OF IgY ANTIBODIES IN EGG YOLK AGAINST β-CASOMORPHIN-7

BY

JOHN YANNAKIS ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

IN

FOOD SCIENCE AND TECHNOLOGY

DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Development of IgY antibodies in egg yolk against β-casomorphin-7 submitted by John Yannakis in partial fulfilment of the requirements for the degree of Master of Science in Food Science and Technology.

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ABSTRACT

A beneficial role to mammalian organisms has been suggested for β-casomorphins, the major bioactive peptides originating from bovine β-casein. Release of β-casomorphins during the manufacture of dairy products would require the development of standard techniques for their detection and quantitation. An immunoassay technique using anti-peptide antibodies raised in the egg yolks of hens can be convenient and cost-effective.

The main objectives of this study were to develop IgY antibody in egg yolks specific for β-casomorphin-7, fragment 60-66 of bovine β-casein, to establish a standard immunoassay protocol and to examine the cross-reactivity of the antibody with other milk proteins.

For the first time antibody against β-casomorphin-7 in egg yolks was produced. β-lactoglobulin, α_{s1}-casein and β-casein did not interfere with the antibody at concentrations lower than 6,250 nmol/l.

In conclusion, the assay might find applications in the detection and quantitation of β-casomorphin-7 in milk products and in its further isolation for commercial utilization.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. ENDOGENOUS OPIOIDS

The term endogenous opioids refers to materials which occur naturally in the brain and other organs and have pharmacological properties similar to those of the opiate substance morphine (1). These are for the most part peptides, mainly precursors or metabolites of the best characterized endogenous opioids, β-endorphin, [Met]-enkephalin and [Leu]-enkephalin (sequences shown in Table 1.1) (1). All of them have a [Met]-enkephalin or [Leu]-enkephalin sequence at their amino terminal regions.

They exert various physiological effects, such as analgesia, regulation of respiration, body temperature, food intake and so on, as shown in Table 1.2. These activities are mediated through the binding to specific receptors of the target cells. The receptors are recognition sites that selectively bind and interact with certain molecules and may respond to such interactions by eliciting a cellular response (2). Opioid receptors have been demonstrated in

the central nervous system, in the autonomic nervous system, in the endocrine system and in the immune system. Depending on the type of ligand they are able to bind, they are called opioid receptors of the μ-, δ-, κ-, ε- or σ-type. Individual receptors are responsible for specific physiological effects: the μ-receptor for analgesia and suppression of intestinal motility, the δ-receptor for emotional behaviour, the κ-receptor for sedation and food intake. Certain ligands are specific for a receptor, like dynorphin which selectively binds to the κ-receptor. However, some ligands have multiple binding abilities for different receptors: [Met]-enkephalin for μ- and δ-receptors, and β-endorphin for μ-, δ- and ε-receptors.

1.2. EXOGENOUS OPIOIDS

1.2.1. OPIOID PEPTIDES DERIVED FROM PLANT PROTEINS

Zioudrou et al. (3) found opioid activity in peptides isolated from peptic digests of wheat gluten, using as assays the inhibition of the contraction of the electrically stimulated mouse vas deferens (MVD) and inhibition of adenylate cyclase of neuroblastoma glioma hybrid cells, as opposed to Huebner et al. (4), who recognized it using the radioreceptor assay.

It has also been reported (5) that the peptic digests of wheat gluten

influence the regulation of gastrointestinal motility and hormone release, especially insulin, and that these effects are inhibited by naloxone. Naloxone is an opiate antagonist of endorphins used to treat morphine addiction (2).

However, information about the structure and the character of these peptides came several years later. Four opioid peptides were isolated from an enzymatic digest of wheat gluten, with structures Gly-Tyr-Tyr-Pro-Thr, Gly-Tyr-Tyr-Pro, Tyr-Gly-Gly-Trp-Leu and Tyr-Gly-Gly-Trp, which were named gluten exorphins A5, A4, B5 and B4, respectively (6). Gluten exorphin B5 showed the most potent activity among these peptides.

Zioudrou et al. (3), also, tested pepsin hydrolysates from a number of plant proteins for opioid activity, as listed in Table 1.3. Grain proteins generally showed stimulatory activity, sometimes mixed with naloxone-reversible activity.

1.2.2. MILK PROTEIN-DERIVED BIOACTIVE PEPTIDES

Milk is a food produced by the mammary glands of the mammal mother so as to provide adequate amounts of all nutrients required by the newborn offspring.

About 20% of human average food protein intake in the United States and the countries of the European Community are milk proteins. They serve as a high quality source of essential amino acids in the nutrition of young children and adults.

It has been suggested (7) that, since peptides are the main degradation products of protein digestion, the relationship between the structure of the

protein and the composition of the peptides released during the digestion process in the gastrointestinal tract should be taken into account in defining the value of a protein. Of particular interest in nutrition and food science are bioactive peptides which are hidden in an inactive state within the protein sequence. These peptides may be released by proteolytic processes *in vivo* and act as modulators of several regulatory functions in the body.

Since 1979 many research groups have searched and found bioactive sequences in milk proteins (Table 1.4). The bioactive fragments were obtained from enzymatic digests (*in vitro* conditions) and/or gastrointestinal digests *(in vivo* conditions) of the appropriate precursor proteins and/or by chemical synthesis of the corresponding bioactive peptides.

According to Teschemacher et al. (9), an exogenous opioid has functional significance in the mammalian organism only if the opioid (or its precursor) is present at a functionally relevant site in the organism, and the compound's presence is correlated with a functionally relevant state or process.

For most of the peptides listed in Table 1.4 there is no evidence so far that their amino acid sequences can be released from their potential precursors in the mammalian organism to exert any functional effect, since they exist as synthetic peptides or their natural structure has been modified by synthesis or isolation procedures. However, the possibility that they might behave as novel types of opioids with agonistic or antagonistic activity cannot be excluded.

Among the milk-related opioids, the β-casomorphins represent the only group for which, so far, evidence in terms of the above has been raised: β-casomorphins or precursors thereof have been demonstrated in the mammalian organism in functionally relevant situations.

1.3. BIOACTIVE SEQUENCES IN MILK PROTEINS

1.3.1. α_{s1}-CASEIN FRAGMENTS

Table 1.5 shows the bioactive peptides derived from bovine α_{s1}-casein. Peptides with opioid activity were isolated from pepsin hydrolysates of bovine α-casein (3) and characterized (10). α-casein exorphin, which corresponds to residues 90-96 of α_{s1}-casein, was the most potent opioid. Synthetic analogues thereof with agonistic activity have been demonstrated to be δ-selective opioid receptor ligands. The peptides proved very resistant to enzymatic degradation. The C-terminal hexapeptide acts as an immunostimulant (11).

Casokinins are inhibitors of the angiotensin-converting enzyme (ACE). This enzyme is responsible for the formation of angiotensin II, the most hypertensive compound known, which reduces blood flow and thereby decreases the renal excretion of fluid and salts. Three such casein-derived bioactive peptides from α_{s1}-casein digests have been described (12).

Meisel et al. (13) purified a caseinophosphopeptide from jejunal chyme of minipigs fed with a casein diet. It was characterized as a nonapeptide corresponding to residues 66-74 of α_{s1}-casein. Three tryptic phosphopeptides were also isolated (14) from *in vitro* digests of whole casein and identified as α_{s1}-casein fragments.

Casoxin D is an opioid antagonist peptide isolated from a peptic-chymotrypsin digest of human α_{s1}-casein, a newly found minor component of human casein, as described by Yoshikawa et al. (15).

1.3.2. α_{s2}-CASEIN FRAGMENTS

Several caseinophosphopeptides corresponding to different regions of α_{s2}-casein were obtained from tryptic digests of sodium caseinate, as shown in Table 1.6 (7).

1.3.3. β-CASEIN FRAGMENTS

Table 1.7 shows the bioactive peptides (other than β-casomorphins) originating from the β-casein molecule.

1.3.4. κ-CASEIN FRAGMENTS

The opioid peptides derived from bovine κ-casein (Table 1.8), named casoxins, were obtained *in vitro* by enzymatic digestion and by chemical

synthesis, respectively. The tetra-, penta- and hexapeptides corresponding to residues 33-38 were modified by methoxylation during the isolation procedure. These peptide type opioid antagonists appear to be μ-selective opioid receptor ligands with relatively low potency as compared to naloxone. They also bind to κ-receptors. Their antagonistic activity has been demonstrated in the guinea pig ileum assay (17).

Chiba et al. (18) synthesized casoxin A and B, fragments of bovine κ-casein, and isolated casoxin C from tryptic digests of bovine κ-casein. All three peptides showed opioid antagonistic activity in the guinea pig ileum assay.

Several κ-casein fragments (casoplatelins) have shown antithrombotic activity (19).

1.3.5. α-LACTALBUMIN AND β-LACTOGLOBULIN FRAGMENTS

Yoshikawa et al. (20) synthesized two tetrapeptides (in the amide form) contained in the primary structures of α-lactalbumin (both human and bovine) and β-lactoglobulin (bovine). The fragment containing residues 50-53 of α-lactalbumin Tyr-Gly-Leu-Phe (in the amide form) was called α-lactorphin, and the 102-105 amide fragment of β-lactoglobulin, Tyr-Leu-Leu-Phe, was called β-lactorphin.

Antila et al. (21) found that these peptides could be released from bovine α-lactalbumin and β-lactoglobulin by *in vitro* proteolysis using different proteolytic enzymes. For α-lactorphin, weak but consistent opioid activity has been demonstrated in opioid receptor assays and in isolated organ preparations, whereas the mechanism of β-lactorphin's stimulatory effect remains unclear (21, 22).

1.3.6. BOVINE SERUM ALBUMIN FRAGMENTS

An opioid peptide with sequence Tyr-Gly-Phe-Gln-Asn-Ala was isolated from a peptic digest of bovine serum albumin (23). This fragment, corresponding to the 399-404 residues of the primary structure of bovine serum albumin, was named serorphin. Serorphin showed a weak opioid activity in the opioid assay systems of mouse vas deferens (MVD) and guinea pig ileum (GPI), being a rather δ-selective ligand.

1.3.7. LACTOFERRIN FRAGMENTS

Lactoferroxins A, B and C were isolated from a peptic digest of human lactoferrin (24); by the isolation procedure all three peptides were methoxylated at the C-terminal. Lactoferroxin A corresponds to human lactoferrin fragment Tyr-Leu-Gly-Ser-Gly-Tyr (residues 318-323), lactoferroxin B to human lactoferrin fragment Arg-Tyr-Tyr-Gly-Tyr (residues 536-540), and lactoferroxin C to human lactoferrin fragment Lys-Tyr-Leu-Gly-Pro-Gln-Tyr (residues 673-679). The

lactoferroxins, like the casoxins, appear to be μ-selective opioid receptor ligands of relatively low antagonistic potency, which has been shown in radioreceptor assays and in the guinea pig ileum preparation.

1.4. β-CASOMORPHINS

1.4.1. STRUCTURE AND NOMENCLATURE

The most thoroughly studied group of milk-derived opioid peptides is a group of peptides isolated from the molecule of bovine β-casein (Figure 1.1) called β-casomorphins (Table 1.9). The first member of the group to be isolated by Brantl et al. (25) was a heptapeptide, which contained tyrosine as N-terminal, was rich in proline, and had an extremely hydrophobic composition. Because of its origin, activity and chain length the heptapeptide was named β-casomorphin-7.

1.4.2. CHARACTERISTICS

Looking at the structure of β-casomorphins, one can observe the following features:

1) The presence of tyrosine residues at their amino termini. This residue in the N-terminal position is essential for the interaction of the respective peptide with opioid receptors. All endorphins so far identified carry tyrosine at the N-terminal

position and any alteration of this residue results in a total absence of bioactivity (29).

2) The presence of another aromatic residue, phenylalanine, in the third position. The β-casomorphins and their D-Ala^2-derivatives are the first natural opioid peptides discovered to possess the amino acid phenylalanine at position 3 from the N-terminal (30).

3) The presence of proline residues in the second, sixth and eighth and, dependent on the species, also in the fourth position. According to Henschen et al. (29), this proline-rich sequence gives them high resistance towards proteolysis.

The sequence of β-casomorphin-7, which corresponds to the sequence 60-66 of bovine β-casein, is also present in the β-casein of other species like ovine (31) and buffalo milk (32), but not in that of rat or mouse (9). Similarly, in human milk casein, the sequence Tyr-Pro-Phe- that makes up the N-terminal of a heptapeptide similar to bovine β-casomorphin-7, that is: Tyr-Pro-Phe-Val-Glu-Pro-Ile (human β-casein 51-57), has been isolated (33) and shown to have opioid activity.

β-casomorphins have been tested for their opioid activities in a variety of assay systems (30). Each of them displayed a much higher potency in the guinea pig ileum longitudinal muscle myenteric plexus preparation (GPI), mainly populated by μ-type opiate receptors, than in the mouse vas deferens preparation (MVD), which contains mostly δ-type opiate receptors. Further, none

of them displayed opioid activity in the isolated rat vas deferens preparation, which contains ε-type opiate receptors. These results clearly suggest that β-casomorphins are μ-type opiate receptor agonists.

β-casomorphin-5 (28, 30) and the amidated tetrapeptide morphiceptin (34) have been found to be the most potent opioid peptides of the group.

β-casomorphins have an intensely bitter taste due to their highly hydrophobic character (29). According to Shinoba et al. (35), β-casomorphin-7 is six times more bitter than caffeine.

1.4.3. DELIVERY OF β-CASOMORPHINS FROM THE DIET

Once the structure of β-casomorphins was elucidated, several questions were asked: what is the nature of their pharmacological activity, are they released from β-casein in the gut, are they absorbed or do they act locally, and what types of activity do they have in the adult and the newborn?

Recent studies have shown that β-casomorphin precursors and β-casomorphins can be released in the gastrointestinal tract of several species including humans. Meisel (36) searched for the presence of β-casomorphins in the duodenal chyme of male adult Gottingen minipigs fed with milk casein. He found a peptide with the sequence of β-casomorphin-11 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu), probably a precursor of

β-casomorphin. This peptide, having a tyrosine residue at the N-terminal, had substantial opioid activity in an opiate receptor-binding assay.

Conducting a similar study in humans, Svedberg et al. (37) examined whether β-casomorphins are released from β-casein after ingestion of cow's milk by healthy adult volunteers. Considerable amounts of β-casomorphin-7 but no β-casomorphin-5 and only small amounts of β-casomorphin-4 or -6 immunoreactive materials were found in intestinal contents at 30 to 150 min after ingestion of milk. Similar results were obtained for *in vitro* digestion of bovine milk which had been designed as a rough imitation of the gastrointestinal digestion process.

The possible release of β-casomorphins in the gastrointestinal tract has been searched for by many groups under *in vitro* conditions. The procedure usually started with subjecting the milk or relatively purified β-casein to the effect of pepsin at pH 2.5 for 30 min to 1 h. In some cases, digestion was made more complete with the stepwise addition of other enzymes to the substrate (38).

Such an example is the addition of pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B (gastric, pancreatic and brush border proteases) and leucine aminopeptidase in buffalo β-casein (32). The result was the release of a putative precursor from β-casein, named procasomorphin (Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Lys) as well as other

peptides, but not β-casomorphin-7.

When β-casein predigested with pepsin was treated with trypsin, trypsin and chymotrypsin or pancreatin, β-casein fragment 59-68, which contains β-casomorphin-7 (fragment 60-66), was obtained (39).

Another attempt for *in vitro* release of β-casomorphins was the one conducted by Yoshikawa et al. (26): β-casein was first digested with thermolysin to give a decapeptide named pro-β-casomorphin-9; further digestion with leucine aminopeptidase gave β-casomorphin-9. This was the first example of the production of β-casomorphins from bovine β-casein by known enzymes.

To obtain information concerning the possibility that release of β-casomorphins might occur in milk on its way from the cow to the consumer, caused by the proteolytic action of microorganisms, cow's milk was incubated with various bacterial species frequently found therein and samples were drawn after various incubation periods (40). High levels of β-casomorphin-7 immunoreactive materials were found in many samples incubated with caseolytic strains.

To what extent are β-casomorphins absorbed from the gut into the blood? This question was addressed in two studies using newborn calves and adult humans.

In the first study (41), blood was collected from newborn calves before

and after their first milk intake after birth. No β-casomorphin-like material was detected before milk ingestion. However, a β-casomorphin-7 precursor was detected in the plasma after milk-feeding. This precursor is a longer molecule than the β-casomorphin-7 peptide. Although this precursor proved resistant to enzymatic degradation during a 30 min incubation in the plasma of newborn calves, β-casomorphin-7 is degraded under these conditions. The formation of a precursor of β-casomorphin-7, which is protected from degradation in the blood, suggests that these peptides may reach different target organs and exert their function. The degradation of β-casomorphins in plasma is very likely because of the presence of dipeptidyl peptidase IV enzyme, which cleaves dipeptides from the N-terminal end of the peptide when proline is penultimate from the free amino terminal. Thus, extension of the peptide beyond the N-terminal would protect these peptides from degradation. Indeed, β-casomorphin analogs, in which the proline residue in position 2 has been replaced by D-Ala^2 are completely stable in plasma, further supporting the role of dipeptidyl peptidase IV in β-casomorphin degradation (30). The presence of this enzyme has been described in several organs in humans such as the submaxillary gland, kidney, placenta (42).

The second study (43) gave different results. Within 30 to 120 min after feeding milk, buttermilk, or cheese to healthy human adults, no β-casomorphins could be detected in the plasma.

1.4.4. PHYSIOLOGICAL ACTIONS OF β-CASOMORPHINS

It is not yet clear how much of the ingested milk β-casein does eventually give rise to β-casomorphins and whether the latter's actions are restricted to the bowel or whether β-casomorphins penetrate the blood and subsequently enter the central nervous system or other organs to elicit opioid effects. However, there is strong evidence for a variety of physiological effects, as described in the next paragraphs.

1.4.4.1. GASTROINTESTINAL TRACT

Kromer et al. (44) found that casein-derived peptides, such as β-casomorphin-5, influence the peristaltic activity of the ileum. The opioid agonists act to decrease peristalsis, an effect blocked by naloxone.

More recently, Daniel et al. (45) looking for a modulatory role of a β-casomorphin analogue on several gastrointestinal functions in rats, showed that this peptide does affect gastric acid secretion and pancreatic exocrine secretion when given intravenously and gastrointestinal motility when given by gastric tube. To explain its possible effects on gastrointestinal functions, the peptide would need to be absorbed as an intact molecule across the intestinal mucosa, because no luminal gastrointestinal receptors have been identified so far (46). Although lack of transfer of β-casomorphins and analogues across

the rabbit ileum has been reported (47), rapid absorption of a β-casomorphin analogue from the lamb small intestine has been described (48).

A clinical application of bovine β-casomorphins for treatment of calf diarrhoea has been suggested (49). Calves were quickly treated after being given β-casomorphin-4 amide dissolved in milk. Similar conclusions came from Schulte-Frohlinde et al. (50) when they gave β-casomorphin-4-amide to healthy humans to drink. The peptide had a potent effect on the prolongation of intestinal transit, so a possible therapeutic usefulness of β-casomorphins in the treatment of diarrhoea in man seems justifiable. Finally, reduction of motor activity by β-casomorphin-4 amide was shown in dogs with short bowel syndrome (51).

1.4.4.2. REGULATION OF FOOD INTAKE

Schusdziarra et al. (52) found that ingestion of a meal containing bovine casein peptone or of synthetic β-casomorphins stimulated insulin release in dogs. The stimulating effect was reversed by oral administration of naloxone. Similar studies in dogs showed that β-casomorphins or milk lead to an increase in the release of somatostatin (53) and pancreatic polypeptide (54) above that of a test meal alone. Naloxone prevented this effect, as in the case of insulin release. These studies suggested that active β-casomorphins are released from the ingested milk and reach their target (μ-receptors) in active

form.

Schulte-Frohlinde et al. (50) examined the effect of β-casomorphin-4-amide on postprandial levels of insulin, glucagon, pancreatic polypeptide and glucose in man. The data demonstrated that in man the postprandial effects of milk on pancreatic endocrine function are less likely to be mediated by casomorphins than in dogs.

1.4.4.3. CENTRAL NERVOUS SYSTEM EFFECTS

In order to elicit central nervous system (CNS) effects, β-casomorphins should first penetrate the gastrointestinal barrier and then the blood-brain barrier (55). It has previously been documented that, although β-casomorphins are known to be highly resistant to proteolytic enzymes, inactivation is rapid due to degradation by dipeptidyl-peptidase IV, which is present in the intestinal mucosa and meconium, several tissues like placenta, exocrine glands and kidney, as well as in plasma. Taking into consideration that immunoreactive β-casomorphin material was not detected in the blood of adult human volunteers after milk intake (43), whereas such opioids were detected in newborn calves' plasma after their first milk intake after birth (41), it seems that peptide sequences with potential opioid activity may be able to penetrate the gastrointestinal wall of newborn mammals. Thus, in the form of a peptidase-resistant precursor, they can survive in blood and reach their putative brain receptors. They must cross the epithelial barrier in the intestine to

produce an opioid effect, which has been demonstrated for β-casomorphin analogues (56). After absorption from the gastrointestinal tract, they could cross the blood-brain barrier, taking advantage of the fact that the infant's central nervous system is not fully developed. It has been shown that the blood-brain barrier of infants is immature at the time of birth and up to six months of age (57). Besides, there is evidence that under certain conditions oligopeptides or even large proteins are able to penetrate the brain (58).

When β-casomorphins were orally administered in newborn rats (59), they could induce modification in μ-receptors in the brain, suggesting that they might have a modulatory effect on brain development and thus be biologically active peptides in the first stages of life.

A study conducted by Panksepp et al. (60) showed that β-casomorphins can also have modulatory effects on social behaviour during early development. Intraventricular administration of β-casomorphins significantly reduced separation-distress vocalization in young chickens, an effect which lasted for 30 min and was partially antagonized by naloxone.

Aside from their suggested role in the infant-mother relationship, the effect of β-casomorphins on the most common behaviour during the postnatal period, sleep, has been examined on young rats (55). Significant alterations in sleep, reversible by naloxone, were caused when a relatively high dose of β-casomorphin was administered.

1.4.4.4. EFFECTS DURING PREGNANCY AND LACTATION

β-casomorphins or their precursors have been detected in the plasma of women during pregnancy and after delivery, whereas such material was not found in the plasma of men or non-pregnant women (61). As opioid peptides, β-casomorphins might exert a modulatory effect on prolactin or oxytocin release (hormones involved in the regulation of milk production and ejection during lactation), when released from the mammary gland (62). Indeed, an increase of the prolactin concentration observed when bovine β-casomorphin-7 was administered in rats (63) is compatible with this hypothesis.

In addition, a pathophysiological role of β-casomorphins has been postulated for the female organism, as well. A report by Lindstrom et al. (64) linked high levels of β-casomorphins in the central nervous system to post-partum psychosis. Post-partum psychosis occurs in 1 to 2 per 1,000 women within 6 months after delivery. Though the cause of this phenomenon is unknown, a connection between milk production and the onset of psychosis has been suggested. A study by Lindstrom et al. (64) indicated that this condition might be associated with the presence of these human milk casein-derived peptides in the cerebrospinal fluid after parturition.

1.4.4.5. β-CASOMORPHINS AND RESPIRATION

β-casomorphins have been shown to have a potent central respiratory depressive effect. When administered to adult rats or to newborn rabbits, β-casomorphin-5, -7, and morphiceptin caused dose-related, naloxone-reversed depression of ventilatory frequency and tidal volume (65). Morphiceptin and β-casomorphin-7 were as potent as morphine, whereas β-casomorphin-5 was 10 times as potent. All agents decreased inspiratory time and prolonged the expiratory phase by delaying the set point for inspiration. Only morphiceptin was active after systemic administration; the other compounds were active only after intracerebroventricular injection.

"Sudden Infant Death Syndrome" (SIDS) is the leading cause of death in infants between one month and one year of age. The most commonly accepted cause of this syndrome is apnea. It is suggested that these infants are disposed to respiratory apnea because of abnormal autonomic nervous system development and respiratory control mechanisms, and opioid peptides derived from milk might play a role (66), a suggestion supported by the fact that the syndrome appears to be practically absent among oriental children fed on soya milk and their substitutes. This hypothesis has further been enhanced by the results of a study performed in the human infantile brainstem, where β-casomorphin-8 immunoreactive material was detected, as well as β-endorphin (67).

1.4.4.6. β-CASOMORPHINS AND THE IMMUNE SYSTEM

Several immunostimulating peptide fractions from casein have been identified. The immunopeptide derived from β-casein represents the C-terminal part of β-casomorphin-11. Immunopeptides have been shown to stimulate the phagocytic activities of murine and human macrophages, and to protect against *Klebsiella pneumoniae*. These peptides exert their action possibly by stimulating the proliferation and maturation of T cells and natural killer cells for the defence of the newborn against a large range of bacteria, particularly enteric bacteria (68).

1.5. BIOACTIVE PEPTIDES AND FOOD PROCESSING

Exciting research results in the field of bioactive peptides, regarding their possible physiological significance and potential commercial utilization, offer new prospects for evaluating the quality of proteins and food. This provides another reason why the technological treatment of food deserves particular attention. Structural and chemical changes taking place during the processing of food proteins may affect the profile of bioactive peptides produced during intestinal digestion. Since digestive enzymes react with structurally altered proteins as if they were different substrates, the processing treatment can influence gastrointestinal proteolysis. Indeed, this has been shown in

experiments where the supply of peptides to the small intestine was related to the milk processing technology used (69).

As clearly stated previously, resistance to enzymatic hydrolysis is a common characteristic among most bioactive peptides, mainly because of the high content of organically bound phosphate groups or prolyl residues, as in the case of β-casomorphins. The possible formation of additional indigestible peptide sequences during food processing deserves special attention, since this may promote both the formation and absorption of bioactive peptides that do not occur naturally in the precursor protein. It has been found that heat and/or alkali treatment can generate hydrolysis-resistant intermolecular and intramolecular covalent bonds (70). Such processing conditions promote especially the formation of lysinoalanine and the racemic conversion of L- to D-amino acids and, consequently, the formation of indigestible peptide bonds. Further possible chemical changes of amino acid side chain groups and peptide bonds during processing are desulfonation, oxidative changes, hydrolysis, pyrolysis and radiolysis (71).

Bioactive peptides may also be released during the manufacture of several dairy products, and may be ingested as food components. It is known that partially hydrolysed milk proteins for hypoallergenic infant formulas and for clinical applications in enteral nutrition consist exclusively of peptides. Results from a study conducted by Stumer et al. (72) confirmed the hypothesis that infant formulas with predigested casein as a protein source contain large quantities of β-casomorphin-like peptides compared to cows' or soy milk

formulas. Moreover, secondary proteolysis during cheese ripening leads to the formation of various peptides. Indeed, as has been previously described (40), a number of caseolytic bacteria used in the production of cheese and other dairy products can release β-casomorphin-like substances.

Many known bioactive peptides generated during processing and/or digestion of native or processed milk proteins are very potent and even extremely low amounts might be sufficient to exert physiological effects. The liberated peptides may produce local effects on the gastrointestinal tract after intestinal absorption, or they may enter the blood stream intact and reach peripheral organs. However, it is difficult to measure the absorption of bioactive peptides, as they cannot be easily detected in plasma by chemical methods, and, further, are rapidly degraded once they have entered the blood stream. A half-life of 15-25 min for β-casomorphins in rat plasma has been found (30).

1.6. A COMMERCIAL ROLE FOR BIOACTIVE PEPTIDES

The physiological role of bioactive peptides as exogenous 'food hormones' is not yet fully understood. Furthermore, it is evident that not all the peptides originating from food proteins necessarily produce beneficial effects: some peptides could have a pathophysiological role if they interfered with

normal regulatory processes in the body. Nevertheless, several bioactive peptides derived from milk proteins have been shown to exert beneficial physiological effects. Such findings could represent an additional nutritional benefit for milk .

Interesting applications have been found for some casein-derived peptides, with caseinophosphopeptides being a good example. It has been shown that they inhibit caries lesions through recalcification of the dental enamel and hence can be used in the treatment of dental disease. A proposed role for this group of bioactive peptides is to use as pharmaceutical preparations such as tablets, toothpaste and dental filling material, as well as dietary products like bread, cake and chewing gums (8). Besides, since cheese contains phosphopeptides as natural constituents, a protective role for cheese against tooth decay has also been considered (73).

The following possibilities for the commercialization of bioactive peptides, including β-casomorphins, look very promising, according to Schlimme et al. (8):

1) supplementation of the diet with desirable synthetic bioactive peptides,

2) production of desirable bioactive peptides during food processing using genetically transformed microorganisms,

3) incorporation of the genes coding for the desirable peptides or introduction of new sites susceptible to proteases into the precursor by genetic engineering techniques,

4) administration of bioactive peptides as pharmaceutical preparations.

1.7. RESEARCH OBJECTIVES

Within the last twenty years, several research groups have found evidence which strongly enhances the concept that milk and dairy products offer a variety of physiological benefits to the human. The hypothesis that milk and milk products may provide a large spectrum of peptides that possess biological functions may lead to their commercial application as food ingredients and pharmaceutical, dietetic and prophylactic products.

β-casomorphins, a group of peptides derived from bovine or human casein, may be responsible for biological activity similar to morphine. Extensive research on these peptides started in Europe and is currently being conducted in many countries, with emphasis mainly on the determination of physiological effects, ability to reach receptors and stability *in vivo*.

β-casomorphins larger than those found *in vitro* have been discovered in intestinal contents or plasma, after ingestion of a casein diet or milk, respectively. Since β-casomorphins are a hydrolysis product of β-casein, they may be naturally present in dairy products and ingredients. Degradation of milk proteins occurs in cheese and other fermented dairy products as they undergo proteolysis during ripening. Peptides can also be enzymatically created during

the preparation of infant formulas and functional casein hydrolysate ingredients. Although infant formulas and casein hydrolysates have been investigated for the presence of β-casomorphins, a search for β-casomorphins in other dairy products has so far only been suggested.

To facilitate investigations for the presence of β-casomorphins in milk and other dairy products and their possible physiological effects, it is necessary to develop standard techniques for their detection and quantitation. Enzyme immunoassays using anti-peptide antibodies developed in egg yolks of laying hens have found many applications in food-related areas, due to their advantages over conventional methods, in terms of sensitivity, convenience, cost, ease, and continuity of sample collection.

The main objectives of this study were to:

1) prepare high affinity antibody specific for β-casomorphin-7 from egg yolks,

2) establish a standard curve for β-casomorphin-7 using an indirect competitive ELISA (enzyme-linked immunosorbent assay),

3) examine the cross-reactivity of the prepared antibody with other proteins present in milk.

The development of a reliable ELISA could be further utilized in future studies to examine the presence of the peptide in several dairy products, as well as in the intestinal contents of experimental animals after feeding trials.

Table 1.1. Structures of β-endorphin, [Met] and [Leu]-enkephalin.

Peptides	Structure
[Leu]-enkephalin	Tyr-Gly-Gly-Phe-Leu
[Met]-enkephalin	Tyr-Gly-Gly-Phe-Met
β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Thr-Lys-Lys-Gly-Gln

Adapted from reference 1

Table 1.2. Physiological effects of opioid peptides.

Central effects	Peripheral effects
1. Analgesia	1. Suppression of intestinal motility
2. Catalepsy	2. Potentiation of MSH activity
3. Sedation and torpor	
4. Respiratory depression	
5. Hypotension	
6. Regulation of body temperature	
7. Regulation of food intake	
8. Suppression of gastric secretion	
9. Regulation of hormone levels	
10. Grooming	
11. Regulation of sexual behaviour	

Table 1.3. Plant protein-derived opioid peptides.

Protein	Source
Gliadin	Wheat
Gluten	Wheat
Zein	Maize
Hordein	Barley
Avenin	Oats
Secalin	Rye
α-protein	Soy

Adapted from reference 3

Table 1.4. Milk protein-derived bioactive peptides.

Bioactivity	Bioactive peptides	Protein precursor
Opioid Agonists	α-casein exorphins	α_{s1}-casein
	β-casomorphins	β-casein
	β-casorphins	human β-casein
	α-lactorphins	human α-lactalbumin
		bovine α-lactalbumin
	β-lactorphin	β-lactoglobulin
	serorphin	bovine serum albumin
Opioid Antagonists	casoxins	κ-casein
		human α_{s1}-casein
	lactoferroxins	human lactoferrin
Antihypertensive	casokinins	α, β-caseins
Antithrombotic	casoplatelins	κ-casein, transferrin
Immunostimulants	immunopeptides	α, β-caseins
Mineral carriers	caseinophosphopeptides	α, β-caseins

Adapted from references 8 and 9

Table 1.5. Bioactive α_{s1}-casein fragments.

Bioactive peptides	Region in the structure of α_{s1}-casein
α_{s1}-casein exorphins	
Arg-Tyr-Leu-Gly-Tyr-Leu-Glu	f90-96
Arg-Tyr-Leu-Gly-Tyr-Leu	f90-95
Tyr-Leu-Gly-Tyr-Leu-Glu	f91-96
immunopeptides	
Thr-Thr-Met-Pro-Leu-Trp	f194-199
casokinins	
Phe-Phe-Val-Ala-Pro	f23-27
Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	f23-34
Thr-Thr-Met-Pro-Leu-Trp	f194-199
caseinophosphopeptides	
Asp-Ile-Gly-SerP-Glu-SerP-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys	f43-58
Gln-Met-Glu-Ala-Glu-SerP-Ile-SerP-SerP-Glu-Glu-Ile-Val-Pro-Asn-SerP-Val-Glu-Gln-Lys-His	f59-79
Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-SerP-Ala-Glu-Glu-Arg	f106-119
SerP-SerP-SerP-Glu-Glu-Ile-Val-Pro-Asn	f66-74
casoxin D	
Tyr-Val-Pro-Phe-Pro-Pro-Phe	fragment of human α_{s1}-casein

Adapted from references 7, 8, and 9

Table 1.6. Bioactive α_{s2}-casein fragments.

Bioactive peptides	Region in the structure of α_{s2}-casein
caseinophosphopeptides	
Asn-Thr-Met-Glu-His-Val-SerP-SerP-SerP-Glu-Glu-Ser-Ile-Ile-SerP-Gln-Glu-Thr-Tyr-Lys	f2-21
Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-SerP-SerP-SerP-Glu-Glu-SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys	f46-70
Glu-Gln-Leu-SerP-ThrP-SerP-Glu-Glu-Asn-Ser-Lys	f126-136
Thr-Val-Asp-Met-Glu-SerP-Thr-Glu-Val-Phe-Thr-Lys	f138-149

Adapted from references 7, 8, and 9

Table 1.7. Bioactive β-casein fragments.

Bioactive peptides	Region in the structure of β-casein
casokinins	
Ala-Val-Pro-Tyr-Pro-Gln-Arg	f177-183
Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg	f193-202
immunopeptides	
Pro-Gly-Pro-Ile-Pro-Asn	f63-68
Leu-Tyr-Glu	f191-193
caseinophosphopeptides	
Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SerP-Leu-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys	f1-28
Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SerP-Leu-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys	f2-28
Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SerP-Leu-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Arg	f1-25
β-casorphin	
Ile-Tyr-Pro-Ser-NH2 or Tyr-Pro-Ser-Phe-NH2	human f40-43 or f41-44

Adapted from references 8 and 16

Table 1.8. Bioactive κ-casein fragments.

Bioactive peptides	Region in the structure of κ-casein
casoxins	
Ser-Arg-Tyr-Pro-Ser-Tyr (casoxin 6)	f33-38
Arg-Tyr-Pro-Ser-Tyr (casoxin 5)	f34-38
Tyr-Pro-Ser-Tyr (casoxin 4)	f35-38
Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg (casoxin C)	f25-34
Tyr-Pro-Ser-Tyr-Gly-Leu-Asn (casoxin A)	f35-41
Tyr-Pro-Tyr-Tyr (casoxin B)	f61-64
	(human:f31-34)
casoplatelins	
Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys	f103-111
Met-Ala-Ile-Pro-Pro-Lys-Lys	f106-112
Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys	f106-116
Asn-Gln-Asp-Lys	f113-116

Adapted from references 8 and 9

Table 1.9. Structure and nomenclature of β-casomorphins.

Nomenclature	Fragment of β-casein	Reference
β-casomorphin-11	f60-70	(13)
β-casomorphin-9	f60-68	(26)
β-casomorphin-8	f60-67	(27)
β-casomorphin-7	f60-66	(25)
β-casomorphin-6	f60-65	(28)
β-casomorphin-5	f60-64	(29)
β-casomorphin-4	f60-63	(28)
β-casomorphin-4-amide (morphiceptin)	f60-63	(27)
β-casomorphin-3	f60-62	(28)

Figure 1.1. β-casomorphins in the primary structure of bovine β-casein.

41 50

Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-

60 70 76

Val-**Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu**-Pro-Gln-Asn-Ile-Pro-Pro

Adapted from reference 8

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CHAPTER 2

DEVELOPMENT OF IgY ANTIBODIES IN EGG YOLK AGAINST β-CASOMORPHIN-7

2.1. INTRODUCTION

Antibodies currently used in research mainly belong to one of the three following categories: mammalian monoclonal antibodies, mammalian polyclonal antibodies, and avian polyclonal antibodies. The species chosen for antibody production have usually been mammals, including guinea pigs, rabbits, mice, sheep, and horses, although avian antibodies have been recognized for several decades. The only avian species from which antibodies are highly defined and easily accessible is the chicken (1).

In common with the colostra of mammals, yolks of bird's eggs provide a supply of antibodies to the newborn against bacteria and viruses (1). This antibody, equivalent to chicken serum IgG, is termed IgY to denote that it is found in the yolk. The main components of the water soluble fraction of egg yolk are livetins (α-, β-, and γ-livetins) and many endogenous enzymes. γ-livetin

(IgY), being the largest molecule in this fraction, is easy to separate from other proteins found therein (2). The IgY molecule is larger than that of human IgG, 170,000 vs. 150,000 (3), with a larger molecular weight of 60,000-70,000 for the heavy chain compared with 50,000 for human IgG heavy chain. The molecular weights of the light chains of IgY and human IgG are similar, with a value of 22,000. According to Parvari et al. (4), chicken Ig has four CH domains, unlike mammalian Ig which has three CH domains.

Several investigators have examined the transfer of antibodies against bacteria and viruses from the circulation of hens to the yolk, and their conclusion was that the antibody levels in the yolk could often be higher than those in the sera of hens (5). It has been estimated that five to six eggs can be produced by a single hen every week, giving a yolk volume of approximately 15 ml/egg, the antibody concentration of which is higher than in the serum (1). Thus, in one week a hen produces egg antibodies equivalent to 90 to 100 ml of serum or 180 to 200 ml of whole blood, whereas an immunized rabbit can provide a much smaller volume of whole blood per week (about 20 ml) when it is repeatedly bled. Only large mammals such as cows or horses can produce more antibodies than a laying hen.

Since the collecting of eggs from a good breed of hens would be a simple procedure, the utilization of yolks as an alternative source of specific antibodies seems practicable. Antibodies are usually purified from the yolk. Several published purification methods can be used for large-scale purification of chicken antibodies (6,7,8). The possibility of purifying large amounts of

antibodies is of specific interest for oral administration or incorporation in the diet of specific antibodies to prevent bacterial infections (7,8).

A primary objective of this study was the development of IgY antibodies in the egg yolks of laying hens against β-casomorphin-7, fragment 60-66 of bovine β-casein. Additional experiments were focused on the search of a suitable form of preservation of the antibody and its stability under different heat treatments, checking temperatures often used in food processing.

2.2. MATERIALS AND METHODS

2.2.1. CHEMICALS

Imject ® Immunogen EDC Conjugation Kit 77102 (Pierce, Rockford, IL, USA) was used for the preparation of the conjugate to be injected to the hens. Freund's complete and incomplete adjuvants, bovine serum albumin (BSA), bicinchoninic acid (BCA) reagent, ammonium sulphate (anhydrous powder), β-casomorphin-7 (purity 99%), peroxidase-labelled rabbit anti-chicken IgG were all obtained from Sigma Chemical Co. (Sigma-Aldrich, Mississauga, ON, Canada). Microtitre plates (Immulon 2) were purchased from Dynatech (Dynatech Laboratories, Chantilly, VA, USA).

2.2.2. COUPLING OF β-CASOMORPHIN-7 TO BSA

2.2.2.1. REAGENT KIT DESCRIPTION

The reagent kit # 77101 from Pierce contained the following reagents and buffer salts, as described in the Pierce instruction manual (9):

1) 5 x 2 mg Imject ® Bovine serum albumin (BSA). BSA was lyophilized from 0.2 ml of 0.1 M 2-(N-morpholino)-ethanesulfonic acid buffer (MES) containing 0.15 M NaCl, pH 4.7.

2) 5 x 2 mg Imject ® Keyhole limpet hemocyanin (KLH). KLH was lyophilized from 0.2 ml of 0.1 M MES containing 0.9 M NaCl, pH 4.7.

3) 10 x 10 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), as conjugation reagent.

4) Conjugation Buffer: 30 ml 0.1 M MES, 0.9 M NaCl, pH 4.7.

5) 10 x 5 ml gel filtration columns containing 0.02% sodium azide (NaN_3) as a preservative.

6) Purification Buffer Salts. 5 vials, each containing sufficient dry blended buffer mix for gel filtration of 2 samples. The buffer mix is for gel filtration separation and stabilization of immunogens. Upon reconstitution with 60 ml degassed deionized water, 1 vial will contain 0.083 M sodium phosphate buffer, 0.9 M NaCl, pH 7.2.

2.2.2.2. PROTOCOL FOR CONJUGATION OF β-CASOMORPHIN-7 TO BSA WITH EDC

The coupling of β-casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) to BSA was performed using a reagent kit from Pierce (reagent kit 77102), according to the protocol described in the manual (9):

1) 2 mg of BSA were reconstituted with 0.2 ml deionized water.

2) 2 mg of peptide to be coupled were dissolved in 0.5 ml of conjugation buffer (0.1 M MES, 0.9 M NaCl, 0.02% NaN_3, pH 4.7).

3) The 0.5 ml of the peptide solution were added to the 0.2 ml of the BSA solution.

4) The peptide/carrier solution was added to one vial of EDC and dissolved by gentle mixing.

5) The mixture was agitated for 2 h at room temperature.

6) The conjugate was purified by gel filtration. The contents of one bottle of the purification buffer salts were dissolved in 60 ml of degassed, deionized water. The top and bottom caps, respectively, were removed from the column and the storage solution was allowed to drain. The column was then washed with 5 ml purification buffer. The peptide/carrier mixture was applied directly to the top of the disc and the eluate was collected. 0.7 ml aliquots of purification buffer were added and each fraction was collected in a separate tube. The absorbance at 280 nm was continuously recorded. The conjugate was the first fraction peak and eluted between fractions 5-8 (elution volumes 4-6 ml) and the unconjugated peptide after fraction 8 (Figure 2.1).

7) The degree of conjugation was estimated by measuring the difference in absorbance at 280 nm before the conjugation and of the pooled peptide fractions from the gel filtration and calculating the percentage decrease in total absorbance.

8) The conjugate fractions were filtered and kept frozen at -20 °C.

2.2.3. IMMUNIZATION PROCEDURE

Six white Leghorn hybrids from the Poultry Unit of the University of Alberta farm were used in this study. The hens were housed in individual cages for immunization and egg production. For primary immunization a volume corresponding to 200 μg of purified conjugate was emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected into the breast muscle (4 sites). Five booster injections with 100 μg of the same conjugate preparation, but with Freund's incomplete adjuvant instead, were given at 2, 4, 6, 8 and 12 weeks after the first injection. Eggs were collected from each hen every day and stored at 4 °C until used. In total, 510 eggs were collected from six hens during 15 weeks.

2.2.4. PREPARATION OF ANTI-PEPTIDE IMMUNOREAGENT

A water soluble fraction (WSF) that included immunoglobulins from egg yolk was prepared according to the methodology of Akita et al. (8). The egg yolk was separated from the egg white and poured into a graduated cylinder without the membrane. The egg yolk was diluted with distilled water (6 times) up to 100

ml and acidified with 0.1 N HCl to pH 5.0. The mixture of water and egg yolk was held at 4 °C until good separation of the two fractions was achieved. This usually occurred within 12 h. After centrifugation at 10,000 *g for 25 min at 4 °C, the WSF was stored at 4 °C until used.

2.2.5. DIRECT ELISA PROCEDURE

The direct ELISA was performed in five steps according to a protocol developed by Meisel (12), as follows:

1) Antigen adsorption: microtitre plates were coated overnight at 37 °C with 0.5 μg/well β-casomorphin-7 in 100 μl 0.05 mol/l carbonate buffer, pH 9.8.

2) Antibody titre: the coated plate was washed three times with phosphate buffer saline-Tween-20 (PBST). To block non-specific binding, the plate was incubated with 200 μl 1% gelatin hydrolysate in PBS for 30 min at 37 °C, and then washed three times with PBST. Immunoactivity of the IgY soluble in water was determined by incubating 1:1000 dilutions of IgY-immunoreagent (100 μl/well) in PBST/0.2% gelatin-hydrolysate for 1.5 h at 37 °C. The plate was then washed five times with PBST.

3) Enzyme conjugate binding to IgY antibodies: 100 μl of rabbit anti-chicken-IgG-peroxidase conjugate, diluted 1:1000 in PBST/0.5% gelatin-hydrolysate, was added to each well, and incubated for 1.5 h at 37 °C. Thereafter, the plate was washed five times with PBST.

4) Enzymatic reaction: substrate for peroxidase consisted of 2,2'-azino-di

(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). 100 µl of the substrate solution (0.04% ABTS and 0.006% H_2O_2 in phosphate-citrate buffer, pH 4.0) was added to each well. After 10 min incubation at room temperature, the reaction was terminated with the addition of 50 µl/well of 1.5% NaF.

5) Absorbance measurement: absorbances were measured at 405 nm against the substrate blank well. Softmax Kinetic Plate Reader for Macintosh Version 2.3 software was used.

2.2.6. PURIFICATION OF ANTI-PEPTIDE IMMUNOREAGENT

The IgY soluble in water fractions from hen # 3 which were isolated between weeks 3 and 7 (from a total of 22 eggs) showed the highest activity with the direct ELISA (Figure 2.2). These fractions were combined and used for the next steps. The combined immunoreagent will be called IgY water soluble fraction (WSF). This IgY-WSF was further purified by precipitation with ammonium sulphate.

Ammonium sulphate (anhydrous powder) was gradually added to the WSF to 50% saturation (1,000 g ammonium sulphate in 2,000 ml WSF) at 20 °C. The solution was gently stirred for 30 min and then, centrifuged at 10,000 * g for 25 min at 0 °C. The resulting precipitate was dissolved in PBS, pH 7.2. This salting-out procedure was repeated once more. The IgY contained in the precipitate is called IgY wet precipitate (WP), and this term will be used in further descriptions.

2.2.7. PROTEIN ESTIMATION OF THE IgY PREPARATIONS

The protein concentration in the IgY-WSF and the IgY-WP were measured using the Bicinchoninic Acid (BCA) method, according to Jenzano et al. (10). The BCA reagent consisted of bicinchoninic acid solution (reagent A) and 4% (w/v) $CuSO_4 \times 5\ H_2O$ (reagent B) (Sigma Chemical Company). 100 µl of sample (at a dilution 1:100 in PBS) was mixed with 2.0 ml of BCA reagent (at a volume ratio: reagent A: reagent B 50:1), and heated at 37 °C for 30 min. Tubes were cooled to room temperature and read at 562 nm. BSA was used as the standard protein.

2.2.8. HEAT STABILITY OF EGG YOLK ANTIBODIES

An IgY-WP solution (diluted 1:1000 in PBS) was heated for 10 min at 30 to 90 °C or for 0 to 60 min at 70 °C. The IgY activity of the heated samples was measured by a direct ELISA mode. The conditions for the direct ELISA were the same as the ones described in section 2.2.6.

2.2.9. STATISTICS

For the statistical analysis of the data (mean, coefficients of variation) Microsoft Excel Version 5.0 software for Macintosh was used.

2.3. RESULTS AND DISCUSSION

Immunogens are molecules that are able to elicit an immune response, whereas antigens are compounds that will react with an antibody but are not necessarily immunogenic on their own. Immunogenicity is directly related to the molecular weight (MW) of the compound. In general, molecules of MW>5000 are good immunogens as long as they are foreign to the recipient animal. Compounds of MW<1000, however, are practically nonimmunogenic. Antibodies can, however, be raised to such small molecules when they are covalently linked to a naturally immunogenic carrier protein (11).

It was necessary to conjugate β-casomorphin-7, a small peptide composed of seven amino acids with a molecular weight of 789, and, accordingly, not immunogenic, to a larger molecule. An easy-to-use and effective kit from Pierce was used for the preparation of the immunogen. The kit provided a choice of KLH and BSA as carriers. BSA was chosen as a carrier. It has good immunogenicity and many functional groups and is widely used as a carrier protein. By contrast, KLH has several antigenic determinants that may compete with the hapten. Meisel (12), using the same kit and a similar procedure for the preparation of the immunogen, obtained a negligible response with KLH and a very strong one with BSA.

The kit uses EDC coupling for the conjugation which is described as a very efficient one (12). The carbodiimide initially reacts with available carboxyl groups on either the protein carrier or peptide to form an active O-acylurea

intermediate. This unstable intermediate reacts with a primary amine to form an amide bond with the release of a soluble urea derivative. Conjugation may occur at either the C- or N- terminal of the peptide or at any carboxyl or amine containing side chains (9). The phenolic group of tyrosine is the functional group for β-casomorphin-7.

In order to confirm that β-casomorphin-7 was conjugated to BSA, the degree of conjugation was estimated. This was done by measuring the difference in absorbance at 280 nm before the conjugation, which was 0.86543, and of the pooled peptide fractions from the gel filtration (fractions 8-14), which was found to be 0.26568. The percentage decrease in total absorbance gave the degree of conjugation. Calculating, a degree of conjugation 69.3% was obtained.

The first peak (Figure 2.1) corresponds to the β-casomorphin-7/BSA conjugate, because this species is retained the least from the gel pores. On the contrary, the unconjugated peptide elutes last, because it is retained the most by the gel.

Six hens were immunized with β-casomorphin-7/BSA conjugate and five booster injections were given at weeks 2, 4, 6, 8, and 12 after the first injection. Not all the hens responded the same against the conjugate. Actually, only two of them (hen # 3 and hen # 5) showed a response and of these the IgY preparations coming from hen # 3, having a significant activity, were chosen for use as follows.

The monitoring of the anti-peptide activity in the egg yolk during a 15-week period was carried out using a direct ELISA mode. For the direct ELISA, a protocol developed by Meisel (12) was followed. The activity obtained with an 1:1000 dilution of the IgY soluble in water preparations is illustrated in Figures 2.2 and 2.3. For all hens, the experiments with the direct ELISA were conducted in triplicate within the same microtitre plate and in triplicate with different microtitre plates. The intra-assay coefficients of variation (intra-assay CV) were between 6.7 and 9.9% and the inter-assay coefficients of variation (inter-assay CV) were between 9.8 and 14.8%, which are satisfactory for such assays (13).

For hen # 3, maximal response was reached six weeks after primary immunization. The IgY preparations isolated between weeks 3 and 7 were combined and used for the next steps. The protein concentration of the IgY-WSF resulting from the combined volume was determined using the BCA method and found to be 545 μg/ml. Its activity is demonstrated in Figure 2.4. The experiments with the direct ELISA measuring the antibody activity of the IgY-WSF as a function of the protein concentration were conducted in triplicate within the same microtitre plate (intra-assay CV 7.7-9.2%) and in triplicate with different microtitre plates (inter-assay CV 10.9-14.9%).

The IgY-WSF was further purified by precipitation with ammonium sulphate at 50% saturation. Salt precipitation causes selective removal of some contaminating proteins, and, besides, it has been found that storage of such a precipitate at 4 °C is a very convenient way of preserving the antibody

activity for long periods without any significant loss (14). The protein concentration of the IgY-WP was determined using the BCA method and found to be 356 μg/ml. There is the same trend for the immunoreactivity as a function of protein concentration of the IgY-WP and that of the IgY-WSF (Figure 2.4 and 2.5). The experiments with the direct ELISA for the IgY-WP were conducted in triplicate within the same microtitre plate (intra-assay CV 9.7-10.5%) and in triplicate with different microtitre plates (inter-assay CV 11.9-15.5%) .

The production of an antibody resistant to pasteurization temperatures (65-71 °C) is of special importance, since it might be incorporated in different types of food as a food ingredient and used as an effective prophylactic product against intestinal infections (8). The results from the examination of the heat stability of the anti-β-casomorphin-7 IgY antibody could be helpful in a future research involving antibodies from egg yolks.

The heat stability of egg yolk (IgY) antibody was measured by standing solutions of the IgY (WP) (1:1000 in PBS) for 10 min at 30, 40, 50, 60, 65, 70, 75, 80 or 90 °C, or for 0, 5, 10, 20, 30 or 60 min at 70 °C. The activities remaining after incubation were expressed as a percentage of the original activity. As shown in Figure 2.6, the antibody activities were found to be stable when standing for 10 min at 30 and 40 °C, more sensitive at the range 60-70 °C, and showing loss of most of the activity above 70 °C. The experiments with the direct ELISA for this part of the research were conducted in triplicate within the same microtitre plate (intra-assay CV 3.7-7.2%) and in triplicate with different microtitre plates (inter-assay CV 8.2-12.9%). After heating at for 20 min

at 70 °C, approximately 50% of the original activity was retained in the IgY (WP), as shown in Figure 2.7. The experiments for the last heat treatment were conducted in triplicate within the same microtitre plate (intra-assay CV 4.2-6.2%) and in triplicate with different microtitre plates (inter-assay CV 8.5-13.9%).

2.4. CONCLUSIONS

In the present study we report for the first time the production of antibodies in egg yolks against β-casomorphin-7 (bioactive sequence 60-66 of bovine β-casein). This is actually the first time that IgY antibodies in egg yolk against such a small peptide are raised. Meisel only has recently described the isolation of IgY antibodies against β-casokinin-10, a synthetic peptide that corresponds to residues 193-202 of bovine β-casein (12).

Hen to hen variation or poor immunization techniques might be blamed for the fact that only one hen revealed a very good response. The use of a carrier protein other than BSA might be tested, as well. Despite this fact, large quantities of antibodies were obtained, enough to be used in the next steps: development of indirect ELISA and examination of cross-reactivity with other milk proteins.

Purification of the WSF with ammonium sulphate at 50% saturation

resulted in removal of some contaminating proteins with some loss of the antibody activity of the wet precipitate. This may be explained by the protein concentration results: some IgY might have been removed during the precipitation. Further purification of the IgY-WP might include additional steps, like: alcohol precipitation, ultrafiltration or gel filtration; examination for any loss of activity should be carried out, as well. Experiments evaluating the IgY-WP activity after a 9-month storage at 0 °C and further discussion are presented in Chapter 3.

Finally, the results from the examination of the heat stability indicate that heating above 70 °C for more than 20 min might be detrimental for the antibody activity.

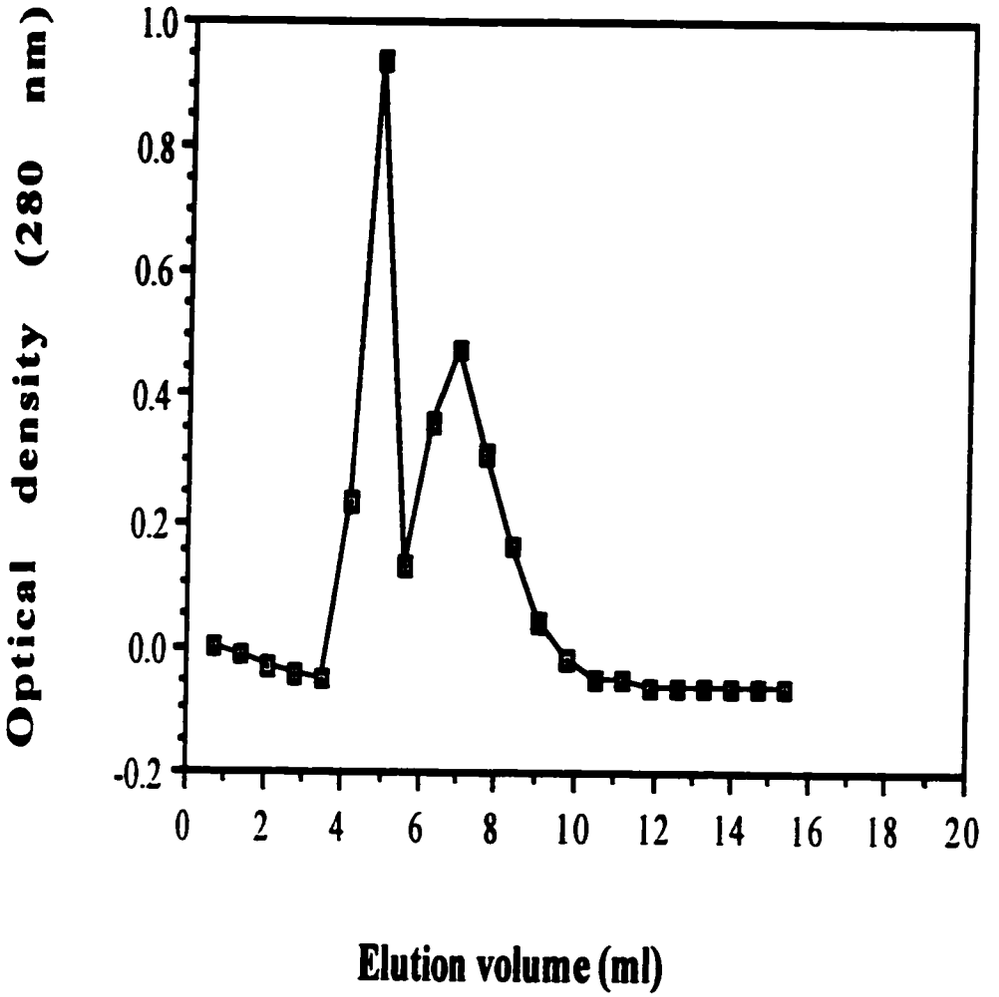

FIGURE 2.1: Monitoring of the conjugation procedure. Recording of the optical density at 280 nm of all fractions (0.7 ml each) eluted from the gel filtration column of the Pierce reagent kit. The β-casomorphin-7/BSA conjugate is the first peak (elution volumes 4-6 ml) and the second peak (elution volumes 6-9 ml) correspond to unconjugated peptide.

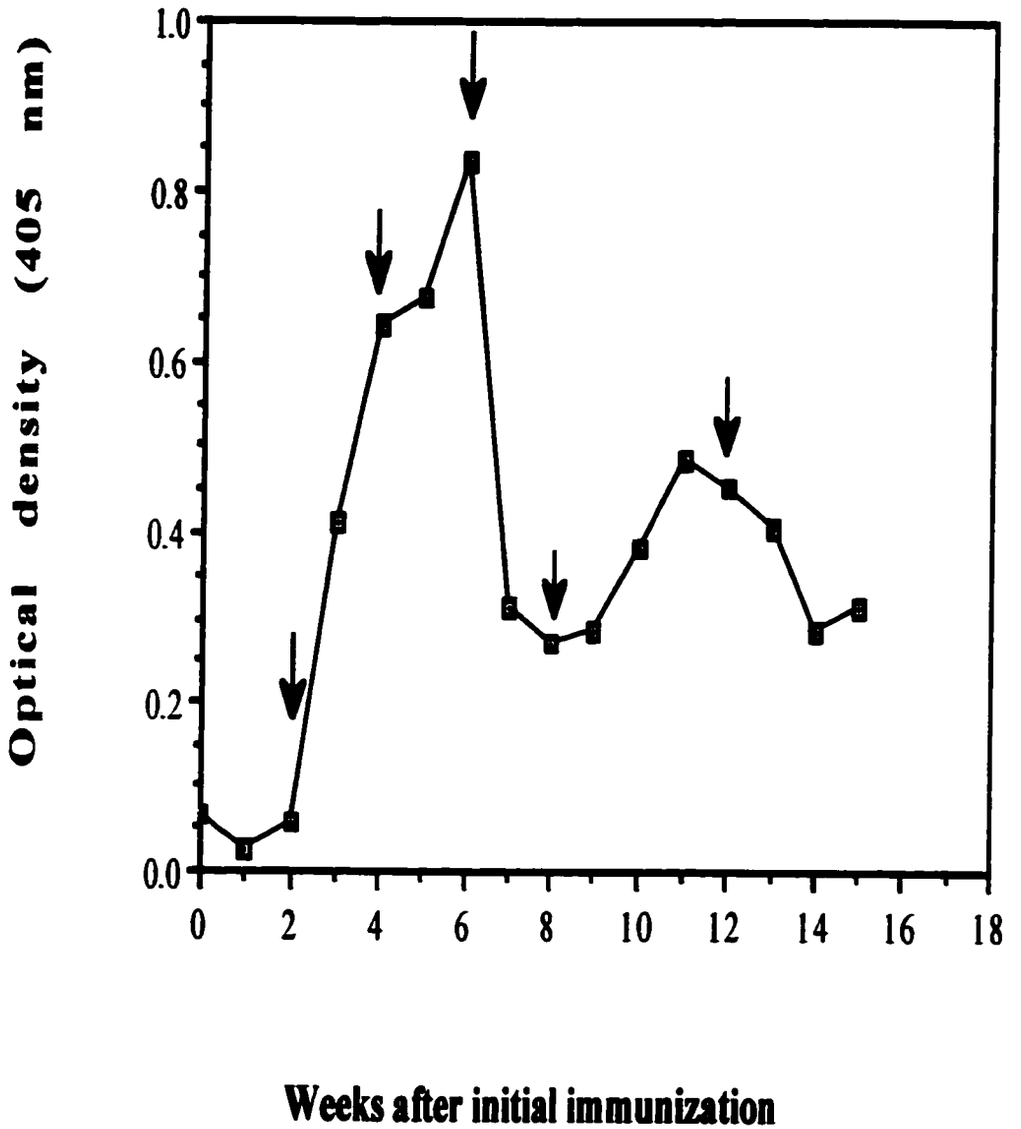

FIGURE 2.2: Changes of antibody levels in water soluble fraction (WSF) from egg yolk (hen # 3) during the 15-week immunization period. Anti-β-casomorphin-7 antibody levels in WSF were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WSF at 1:1000 dilution. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 8.2 and 14.9%. The arrows indicate the booster injection times of hens with the β-casomorphin/BSA conjugate.

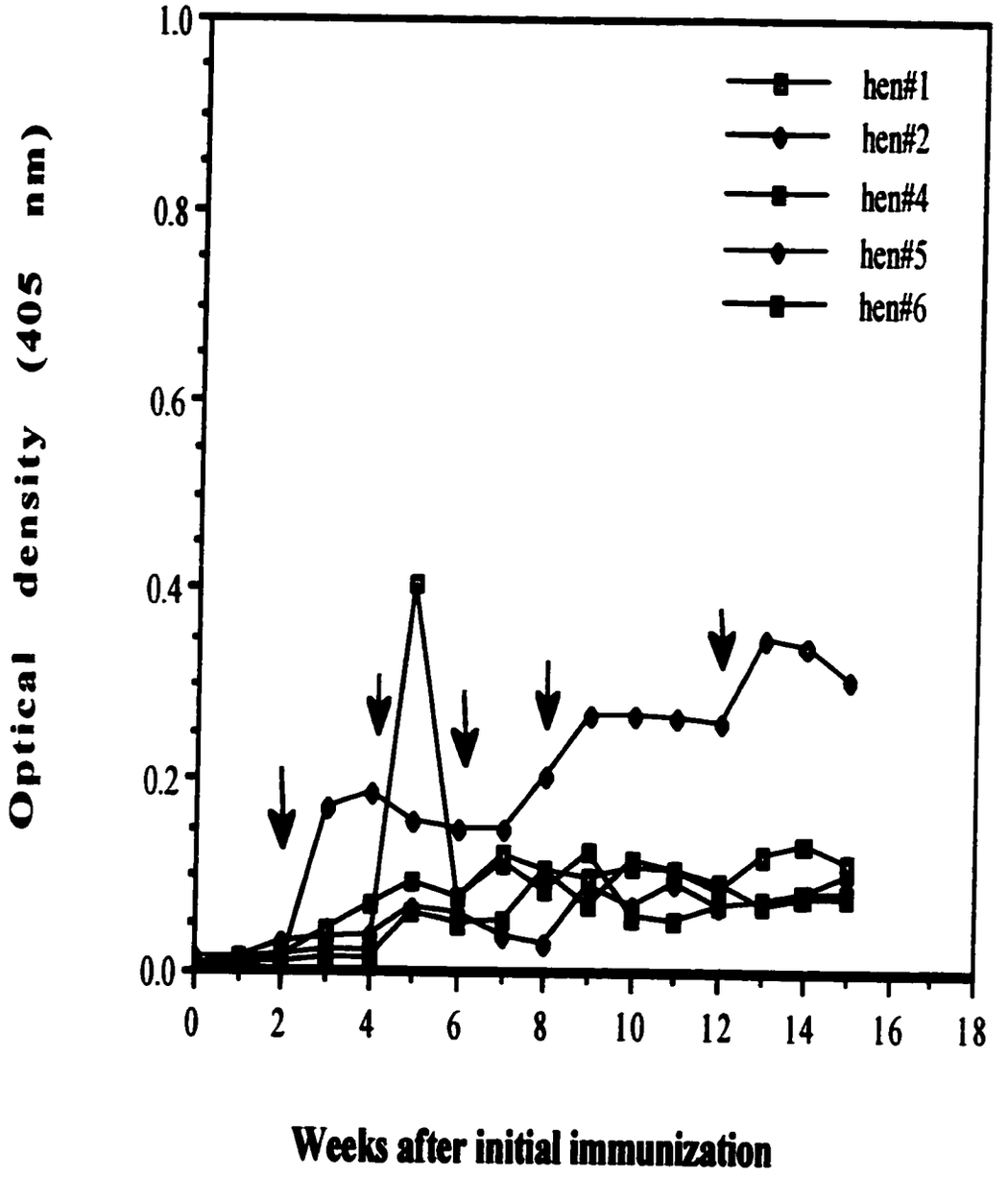

FIGURE 2.3: Changes of antibody levels in water soluble fraction (WSF) from egg yolk (hens # 1,2,4,5,6) during the 15-week immunization period. Anti-β-casomorphin-7 antibody levels in WSF were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WSF at 1:1000 dilution. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 8.6 and 15.1%. The arrows indicate the booster injection times of hens with the β-casomorphin/BSA conjugate.

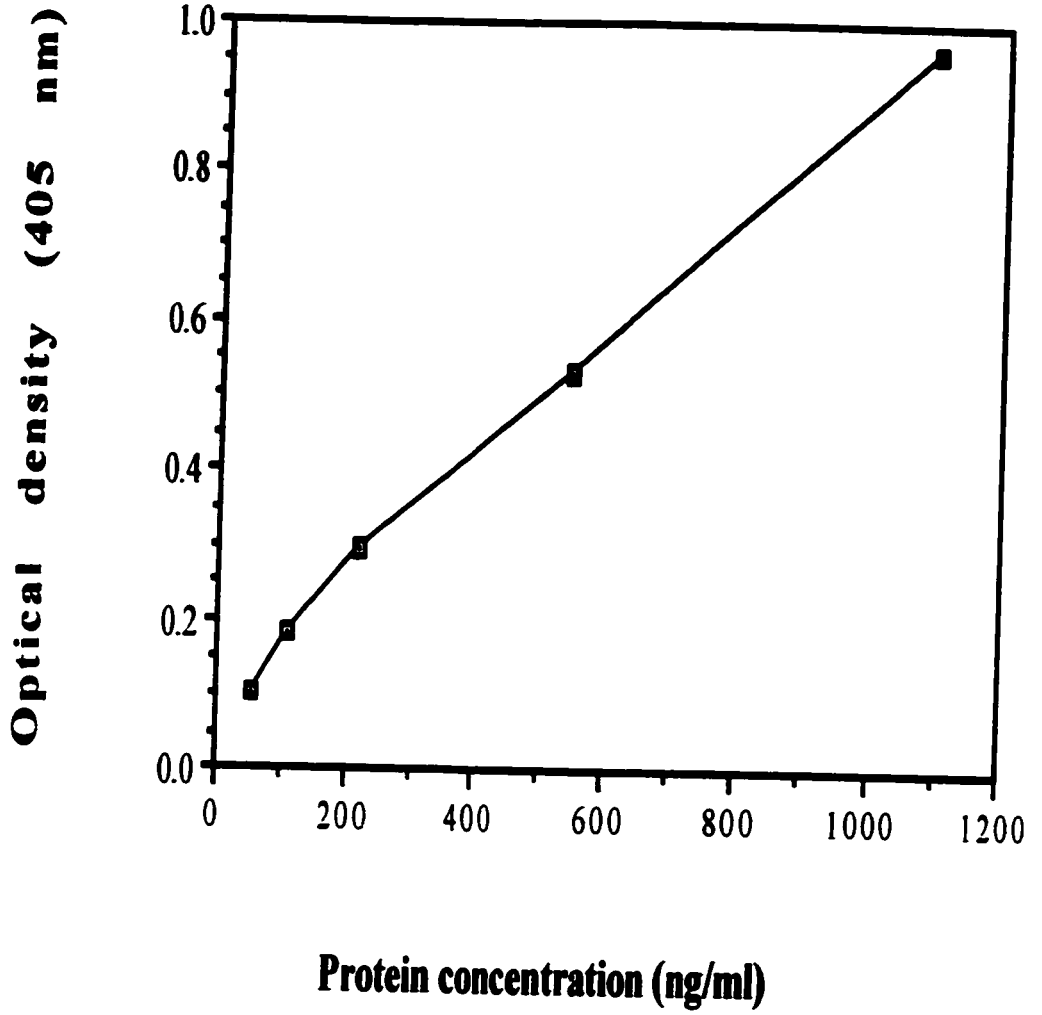

FIGURE 2.4: Response of different dilutions, expressed as protein concentrations, of the IgY included in the water soluble fraction (IgY-WSF). Antibody levels in WSF were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WSF with protein concentrations between 1090 and 54.5 ng/ml. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 8.7 and 14.3%.

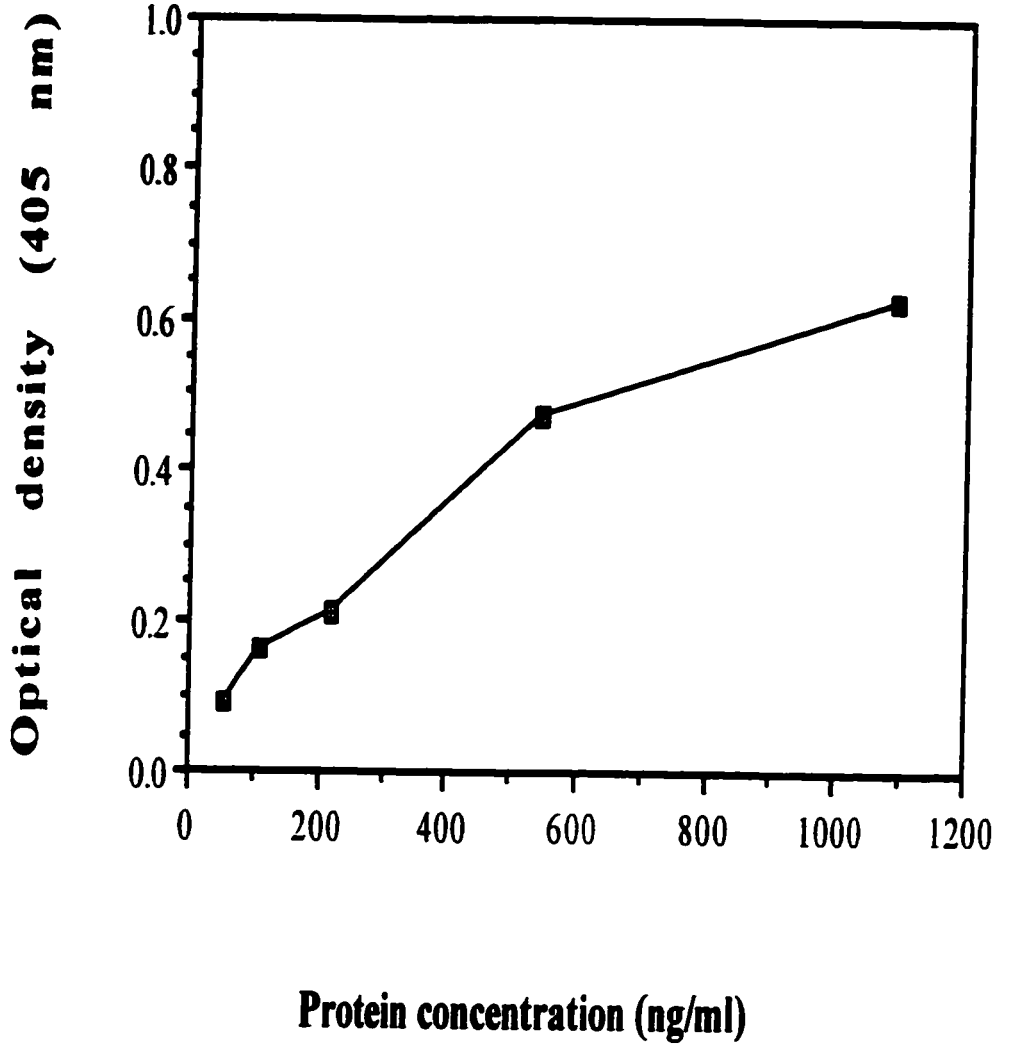

FIGURE 2.5: Response of different dilutions, expressed as protein concentrations, of the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WP with protein concentrations between 1090 and 54.5 ng/ml. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 10.1 and 16.1%.

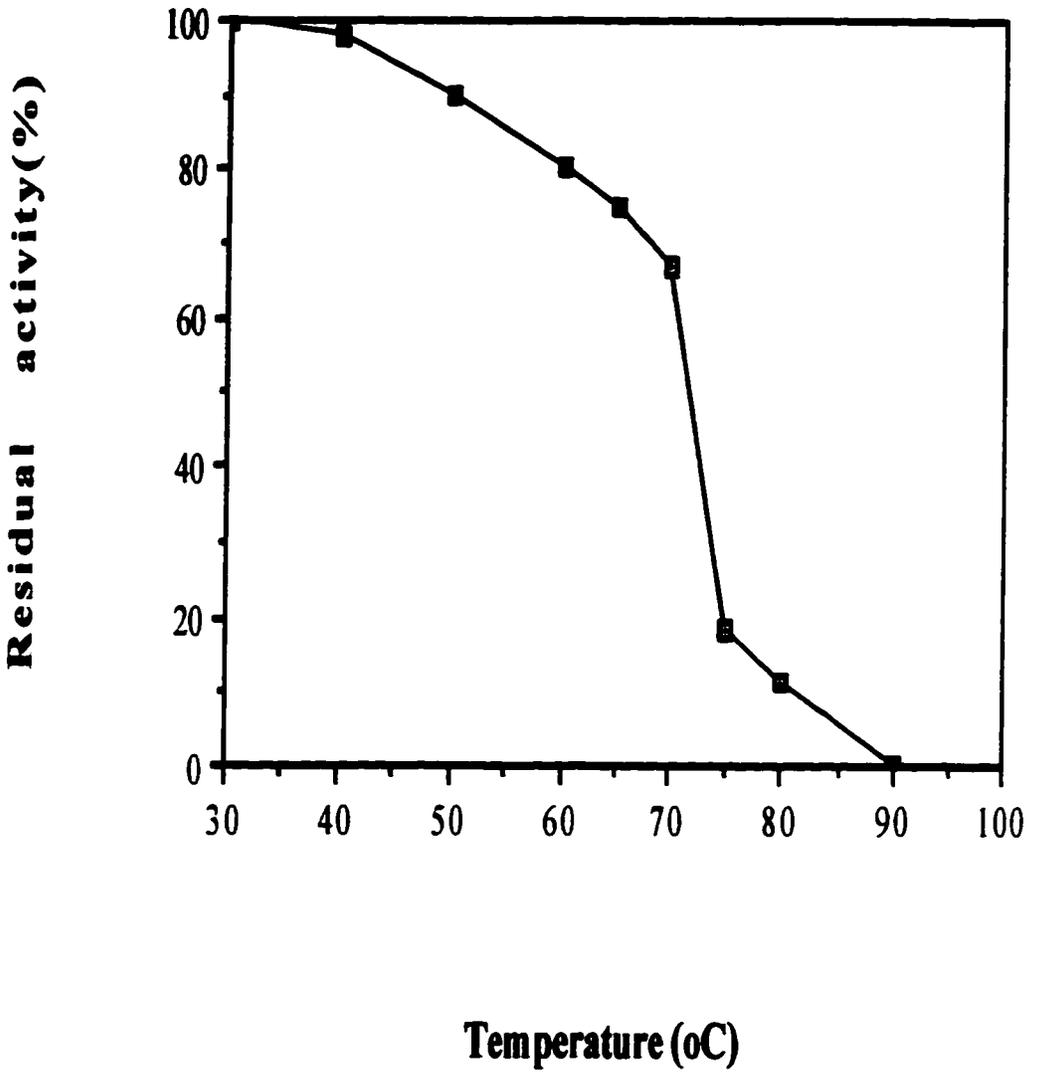

FIGURE 2.6: Heat stability of the IgY included in the wet precipitate (IgY-WP) specific to β-casomorphin-7 incubated for 10 min at various temperatures. Antibody levels in WP were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WP at 1:1000 dilution. Residual activity is defined as: ELISA absorbance (OD 405 nm) at a certain temperature/ELISA absorbance (OD 405 nm) at 30 °C. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 6.4 and 13.0%.

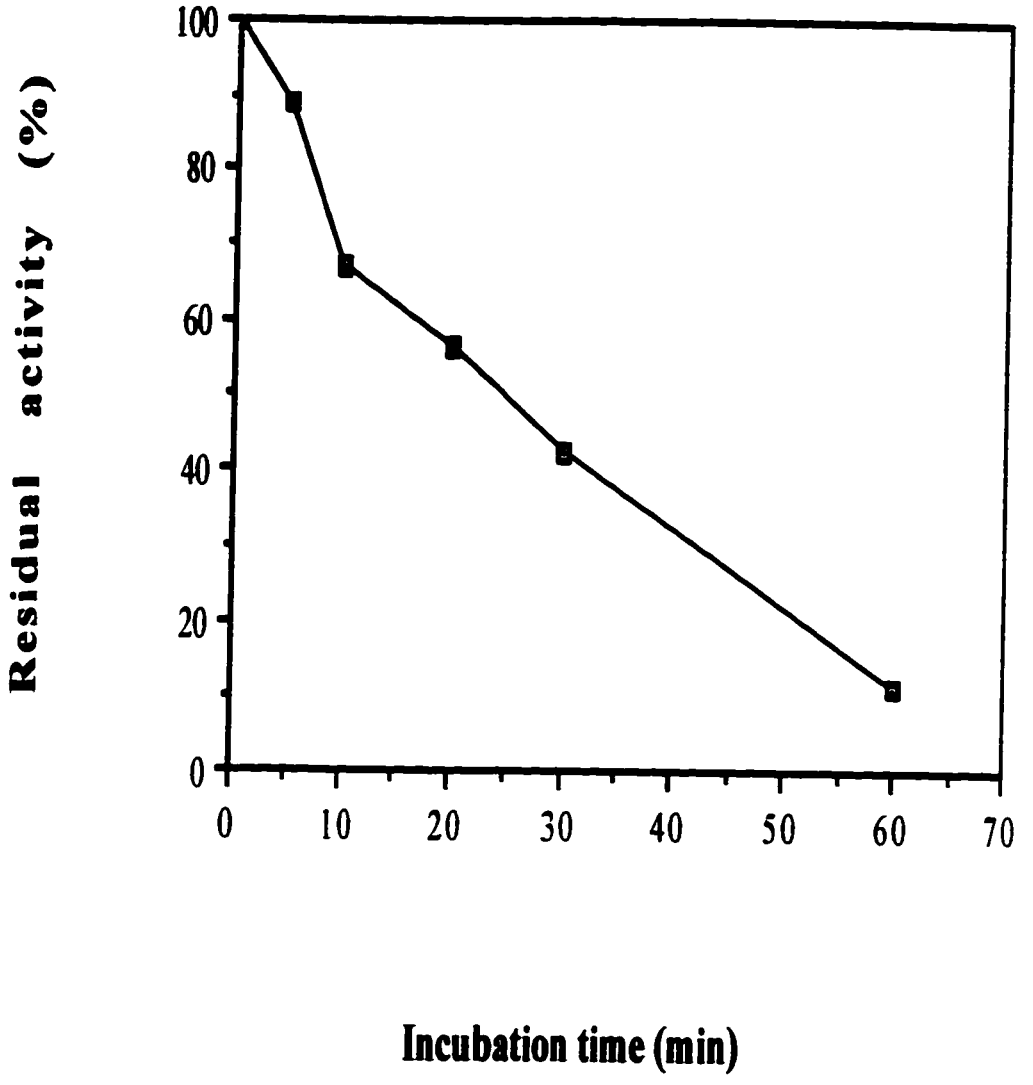

FIGURE 2.7: Heat stability of the IgY included in the wet precipitate (IgY-WP) specific to β-casomorphin-7 incubated at 70 °C for 60 min. Antibody levels in WP were measured using direct ELISA with β-casomorphin-7 as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WP at 1:1000 dilution. Residual activity is defined as: ELISA absorbance (OD 405 nm) at a certain time/ELISA absorbance (OD 405 nm) at 0 min. Values are the mean 9 replicate measurements and the coefficients of variation (CV) were between 5.3 and 11.1%.

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CHAPTER 3

ESTABLISMENT OF STANDARD CURVE FOR β-CASOMORPHIN-7 USING INDIRECT ELISA

3.1. INTRODUCTION

Taking advantage of the highly specific and sensitive nature of immunological reactions, several immunoassay techniques have been developed and applied in the food industry for the detection of naturally occurring constituents, antibiotics, pesticide residues, microorganisms, and fragments of microbial constituents related to food analysis, food production, food processing and food safety (1).

Among the advantages of immunoassays are low cost, fast operation, and increased sensitivity and specificity compared to conventional detection methods, making them useful screening tools for the detection of adulteration and contamination. In Table 3.1. some examples for which immunodiagnostic kits are available commercially or where such potential exists are tabulated.

ELISA has become increasingly useful in food analysis and forensic

chemistry, due to its sensitivity and convenience in assaying antigens and antibodies. One problem with the development of a suitable ELISA is the high cost, particularly when mammalian species are used for the production of antibodies. Such a problem, however, can be easily overcome with the use of immunoglobulins from the eggs of laying hens. Indeed, the cost of feeding and handling is considerably lower for a hen than for a rabbit (2).

The advantages of using the egg yolk as a source of specific antibodies are ease and continuity of sample collection (3). High levels of IgY activity in egg yolk can be maintained for several months by periodic immunization (4). It has been reported that IgY antibodies are stable over time. IgY preparations were stored for ten years at 4 °C without losing their activity (2). Chicken antibodies also retained their activity after six months at room temperature or one month at 37 °C. An additional advantage, and particularly with regard to modern animal protection regulations, is that this system avoids bleeding of animals (3). As well as being painful for the animal, the blood collection procedure is time consuming. Finally, these IgY antibodies can be isolated by simple precipitation techniques (5).

Two applications of IgY antibodies for the development of ELISAs for use in food analysis have recently been reported. The first uses IgY antibodies against soy protein and aims to detect soy protein additives in dairy-like products (6). Similarly, the second one uses IgY antibodies against bovine lactoferrin, which can serve as an indicator protein for cows' milk in the assay of foodstuffs (4).

However, application of immunological methods to the analysis of dairy products is not yet widespread, since only a few antisera against milk constituents are commercially available. However, Meisel (4) was able to assay about 27,000 ELISA plates with up to 38 different samples in duplicate derived from the eggs of a single hen over 28 weeks (a total of 1,000,000 samples); hence it can be concluded that hen eggs provide a sufficient source of high affinity antibodies for use in immunological analysis of dairy products and other foods.

The focus of the present study was to establish a standard curve for β-casomorphin-7 using an indirect ELISA mode and to investigate possible cross-reactivity of the prepared antibody with some milk proteins.

3.2. MATERIALS AND METHODS

3.2.1. CHEMICALS

β-casomorphin-7 (purity 99%) and peroxidase-labelled rabbit anti-chicken IgG were obtained from Sigma Chemical Co. (Sigma-Aldrich Canada, Mississauga, ON, Canada). Microtitre plates (Immulon 2) were purchased from Dynatech (Dynatech Laboratories, Chantilly, VA, USA).

3.2.2. INDIRECT COMPETITIVE ELISA

The indirect ELISA was performed in six steps according to a protocol developed by Meisel (8), as follows:

1) Antigen adsorption: microtitre plates were coated overnight at 37 °C with 0.5 μg/well β-casomorphin-7 in 100 μl 0.05 mol/l carbonate buffer, pH 9.8.

2) Reaction of competitor with IgY-WP immunoreagent: 500 μl competitor at several dilutions in PBST (β-casomorphin-7, α_{s1}-casein, β-casein, or β-lactoglobulin) were mixed with 500 μl IgY-WP (1:1000 dilution in PBST) and incubated overnight at 4 °C under gentle stirring.

3) Antibody binding to sensitized wells: the coated plate (step 1) was washed three times with PBST. To block non-specific binding, the plate was incubated with 200 μl 1% gelatin hydrolysate in PBS for 30 min at 37 °C, and then washed three times with PBST. Following this, 100 μl of the mixture from step 2 were added to each well and the plate was incubated for 1.5 h at 37 °C. The plate was then washed five times with PBST.

4) Enzyme conjugate binding to IgY antibodies: 100 μl of rabbit anti-chicken-IgG-peroxidase conjugate, diluted 1:1000 in PBST/0.5 % gelatin-hydrolysate was added to each well and incubated for 1.5 h at 37 °C. The plate was then washed five times with PBST.

5) Enzymatic reaction: the substrate for peroxidase consisted of 2,2'-azino-di (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). 100 μl of the substrate solution

(0.04% ABTS and 0.006% H_2O_2 in phosphate-citrate buffer, pH 4.0) was added to each well. After 10 min incubation at room temperature, the reaction was terminated with the addition of 50 µl/well of 1.5% NaF.

6) Absorbance measurement: absorbances were measured at 405 nm against the substrate blank well. Softmax Kinetic Plate Reader for Macintosh Version 2.3 software was used.

3.2.3. STATISTICS

For the statistical analysis of the data (mean, coefficients of variation) Microsoft Excel Version 5.0 software for Macintosh was used.

3.3. RESULTS AND DISCUSSION

A checkerboard ELISA (direct ELISA) (Table 3.2) was performed in triplicate using different concentrations of β-casomorphin-7 for coating and different dilutions of the IgY-WP. The purpose of this ELISA was to check if the activity of the antibody was retained after precipitation and to confirm that the conditions (concentration of antigen for coating and dilution of antibody) used during the monitoring of the activity of the IgY from egg yolk were also suitable for the development of the indirect competitive ELISA. In fact, an absorbance value of 0.468 was obtained when 5 µg β-casomorphin-7 for coating and an 1:1000 dilution of antibody were used. The inter-assay coefficient of variation

(inter-assay CV) was between 6.8 and 13.9%.

In addition, a second checkerboard ELISA (Table 3.3) in triplicate, as well, was performed using different concentrations of β-casomorphin-7/BSA conjugate (identical to the one used at the immunization step) for coating and different dilutions of IgY-WP. The only difference from the mode used in the previous experiments was that the IgY-WP was dissolved in PBS/0.1% BSA. The antibody in the egg yolk is actually raised against the β-casomorphin-7/BSA conjugate and not against the peptide only. By dissolving the IgY in buffer containing BSA, a reaction between BSA and the corresponding antibody takes place, but this product of this reaction can be washed away, so the remaining activity is directed against β-casomorphin-7 only. This modification confirmed that there is, indeed, antibody developed against the peptide and this antibody has a greater affinity against the peptide as a part of a conjugate than against the peptide only. The inter-assay coefficient of variation (inter-assay CV) was between 7.8 and 15.3%.The antibody activity against the conjugate is shown in Figure 3.1.

To establish a standard curve for β-casomorphin-7, an indirect competitive ELISA mode was applied. A constant concentration of the IgY-WP (1:1000 dilution in PBST) was incubated with various concentrations of peptide in separate glass tubes. The concentrations used were: 0.05, 0.5, 5, 50, 500, 2500, 5000, 25000 and 50000 ng/ml (Figure 3.2). The experiments for this part of the research were conducted in 5 replicates within the same microtitre plate

(intra-assay CV 6.7-9.2%) and in triplicate with different microtitre plates (inter-assay CV 9.7-16.9%).

The standard curve was linear (r=-0.996) in the range 0.05-500 ng/ml (Figure 3.3) and is described by the equation:

$y = 0.20132 - 0.01160 * x$, where:

x = log (concentration of β-casomorphin-7), and y = optical density (405 nm).

The detection limit was defined as the β-casomorphin-7 concentration corresponding to the absorbance that was 2 standard deviations below the average maximal binding at zero antigen concentration (Bo-2SD) (7). Therefore, the minimum detectable concentration was approximately 5 ng/ml (Bo=0.228 and SD=0.021).

Possible cross-reactivity of the IgY (WP) with other milk protein fractions was also examined. For this purpose, serial 10-fold dilutions from 62,500 to 0.0625 nmol/l (starting from approximately the same molar concentrations used for the indirect ELISA with β-casomorphin-7) of α_{s1}-casein, β-casein and β-lactoglobulin were incubated in separate glass tubes with 1:1000 dilution in PBST of IgY-WP. As illustrated in Figures 3.4-3.7, no cross-reaction occurred with β-lactoglobulin, while, cross-reactivity was found with α_{s1}-casein and β-casein only at concentrations higher than 6,250 nmol/l. An explanation for this might be that the antibody is also able to recognize its epitope in a complex structure, such as the native protein, β-casein. Similar epitopes might also be present in the α_{s1}-structure. Therefore α_{s1}- and β-casein should only interfere

with the assay if present at levels greater than 6,250 nmol/l. The experiments with α_{s1}-casein, β-casein, and β-lactoglobulin were conducted in 5 replicates each within the same microtitre plate and in triplicate with different microtitre plates. The intra-assay CV were 7.7-9.3%, 7.9-10.5%, and 8.1-10.9%, respectively. The inter-assay CV were 9.6-15.8%, 10.6-16.4%, and 11.2-17.1%, respectively.

3.4. CONCLUSIONS

The IgY-WP used for this section of the study had been stored for a period of nine months at 0 °C. According to the literature no significant loss of its activity should be observed (8). However, the ELISA absorbance at 405 nm decreased from 0.428 to 0.228 when an 1:1000 dilution of the IgY-WP and 5 mg/ml of the peptide for coating were used.

Cross-reactivity was found with α_{s1}-casein and β-casein only at concentrations higher than 6,250 nmol/l. This might be attributed to the fact that β-casomorphin-7 may resemble epitopes of the native protein, as well as epitopes of α_{s1}-casein.

Concluding, the developed assay might be used for the detection and quantitation of β-casomorphin-7 in dairy products, if the samples were diluted at a level where no interference occurred.

Table 3.1: Examples of commercially available food-related immunodiagnostic kits.

Food Safety: Bacterial Toxins
Clostridium botulinum neurotoxins A, B, E, F, and G; *Staphylococcus aureus* enterotoxins A, B, C, D, and E
Food Safety: Mycotoxins
Aflatoxins B1, B2, B1 diol, M1 and Q1; ochratoxin; T-2 toxin; 3'-OH-T-2 toxin; T-2 tetraoltetraacetate; HT-2 toxin; group A trichothecenes; roridin A; zearalenone; rubratoxin B; sterigmatocystin; deoxyverrucarol; deoxynivalenol
Food Safety: Pathogenic Microorganisms
Salmonella, *Listeria monocytogenes*, *Escherichia coli*, *Vibrio* spp., *Yersinia enterocolitica*, *Campylobacter jejuni*
Food Safety: Miscellaneous
Mushroom poisoning, algal and seafood toxins, potato glycoalkaloids
Food enzyme / Inhibitor activity
α-amylase, β-amylase, catalase inhibitor, chymotrypsin, debranching enzyme, lipase, malate dehydrogenase, papain, pepsin, polyphenoloxidase, proteolytic enzymes, trypsin, trypsin inhibitor
Interspecies Meat and Adulterant Identification
Beef, sheep, pig, goat, horse, meat products, sausages, processed meats

Adapted from reference 7.

Table 3.2: Checkerboard ELISA using β-casomorphin-7 for coating.

Dilution	Concentration (μg/ml)									
	100	50	25	10	5	2.5	1	0.5	0.25	0.1
500	0.408	b	0.510	0.569	0.623	0.356	0.310	0.257	0.239	0.230
1000	0.154	0.204	b	0.379	0.468	0.352	0.134	0.222	b	0.210
2500	b	0.062	0.131	0.150	0.210	b	0.090	0.147	0.145	0.155
5000	0.061	0.069	0.100	b	0.163	0.082	0.035	0.049	0.043	b
10000	0.020	0.007	b	0.062	0.089	0.031	0.006	b	0.005	b

b: blank

Table 3.3: Checkerboard ELISA using β-casomorphin-7/BSA conjugate for coating.

	Concentration (μg/ml)									
Dilution										
	100	50	25	10	5	2.5	1	0.5	0.25	0.1
1000	2.209	b	2.276	2.282	2.266	2.038	1.756	1.168	0.573	0.176
2500	2.219	2.313	b	2.206	2.218	1.997	1.630	1.031	b	0.139
5000	b	2.115	2.164	2.202	2.072	b	1.423	0.892	0.347	0.103
10000	2.167	2.250	2.066	b	2.046	1.751	1.155	0.689	0.266	b
25000	1.923	1.901	b	1.842	1.710	1.233	0.707	b	0.158	0.012
50000	1.400	1.409	1.377	1.382	1.289	0.806	b	0.263	0.083	0.000

b: blank

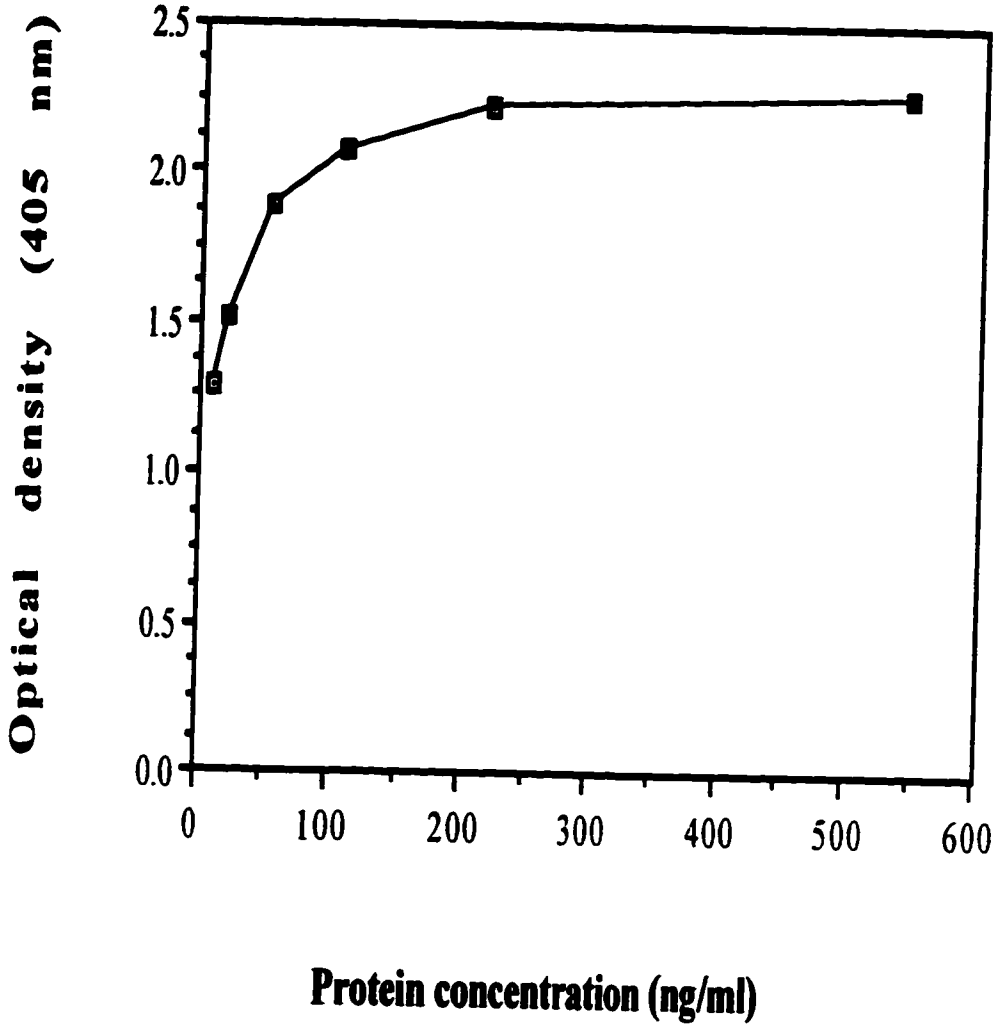

FIGURE 3.1: Response of different dilutions, expressed as protein concentrations, of the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP were measured using direct ELISA with β-casomorphin-7/BSA as an antigen and are expressed as ELISA absorbances (OD 405 nm) for WP with protein concentrations between 545 and 54.5 ng/ml. Values are the mean of 9 replicate measurements and the coefficients of variation (CV) were between 9.2 and 14.7%.

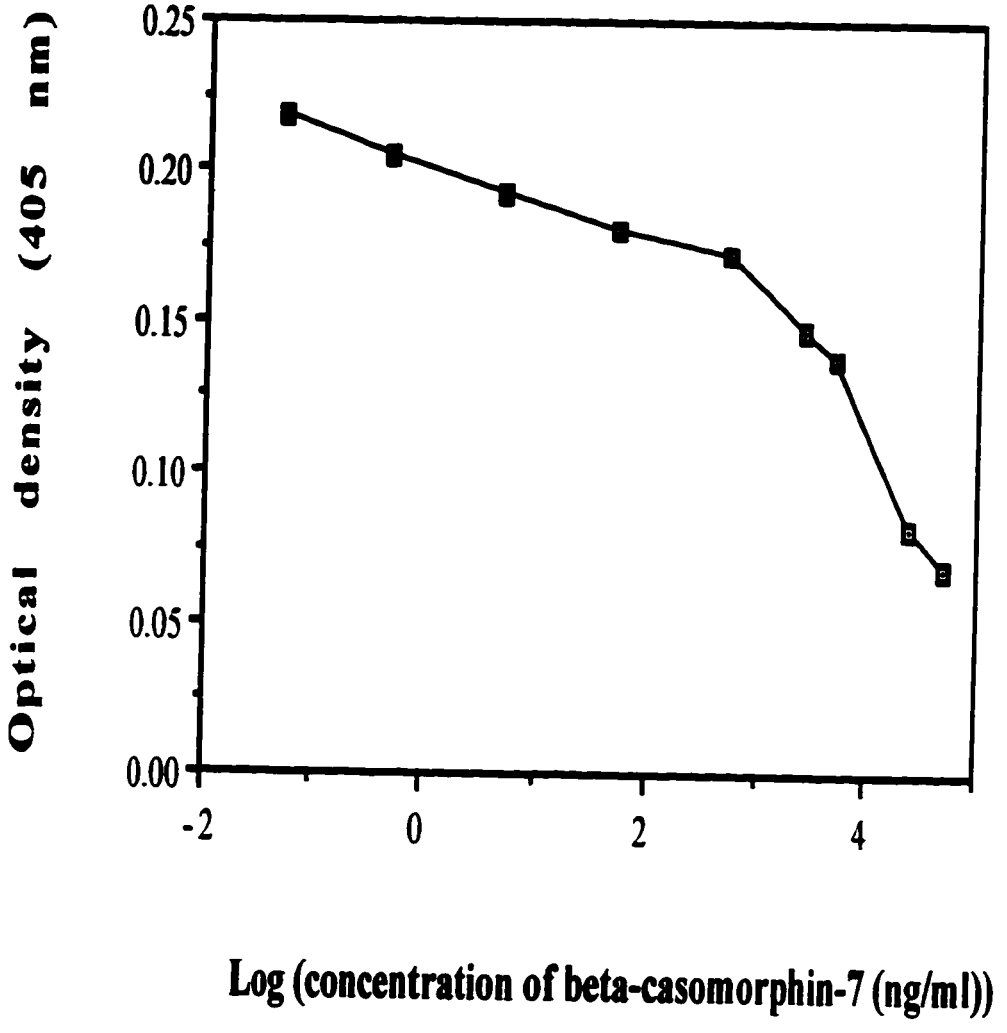

FIGURE 3.2: Indirect ELISA curve using β-casomorphin-7 as competitor. Different concentrations of β-casomorphin-7 are mixed with the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP are expressed as ELISA absorbances (OD 405 nm) at 1:1000 dilution. The data represents the logarithm of the concentration of β-casomorphin-7 (ng/ml) plotted against absorbance. Values are the mean of 15 replicate measurements and the coefficients of variation were between 7.6 and 14.3%.

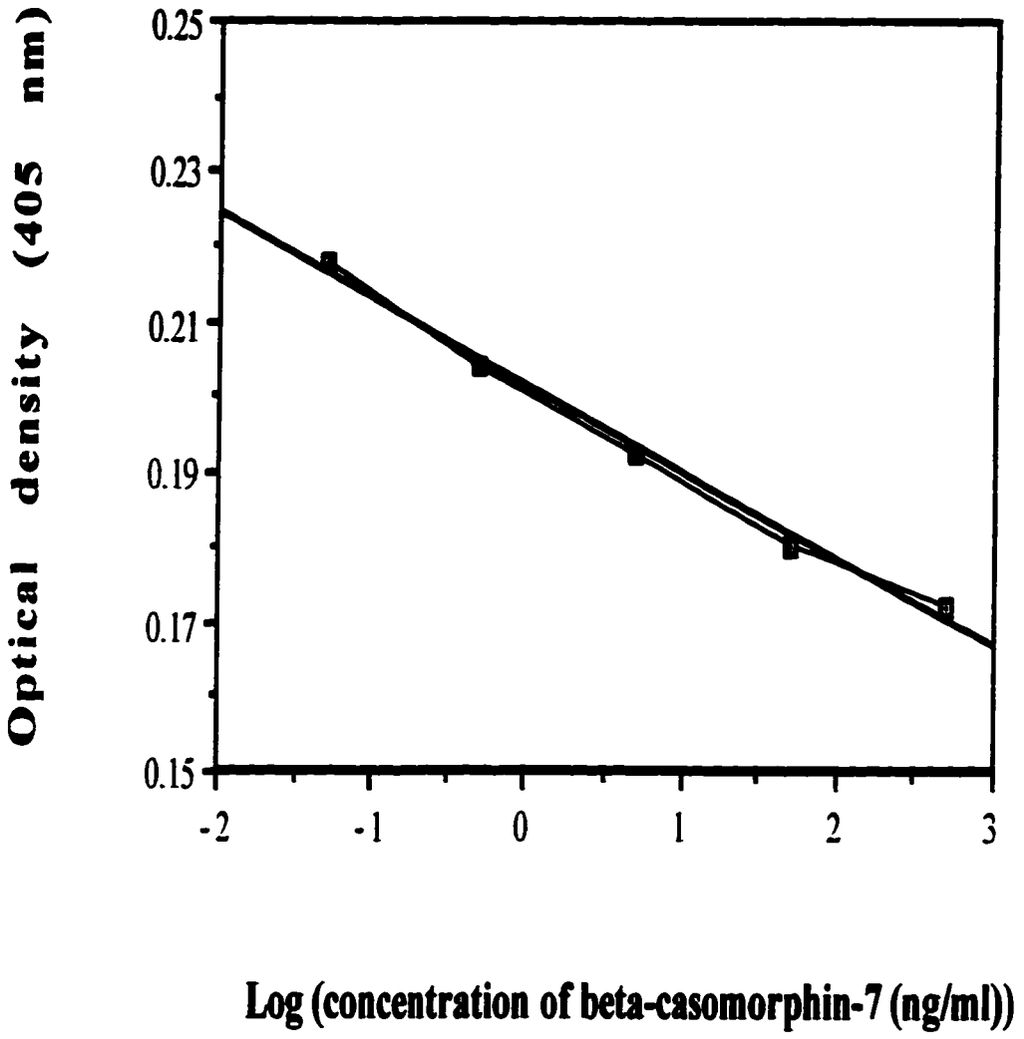

FIGURE 3.3: Standard curve for β-casomorphin-7 using indirect ELISA. Different concentrations of β-casomorphin-7 are mixed with the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP are expressed as ELISA absorbances (OD 405 nm) at 1:1000 dilution. The data represents the logarithm of the concentration of β-casomorphin-7 (ng/ml) plotted against absorbance. The curve is linear (r=-0.996) at the range 0.05-500 ng/ml. The curve for β-casomorphin-7 is described by the equation :

$y = 0.20132 - 0.01160 * x$, where:

x = log (concentration of β-casomorphin-7), and y = optical density (at 405 nm).

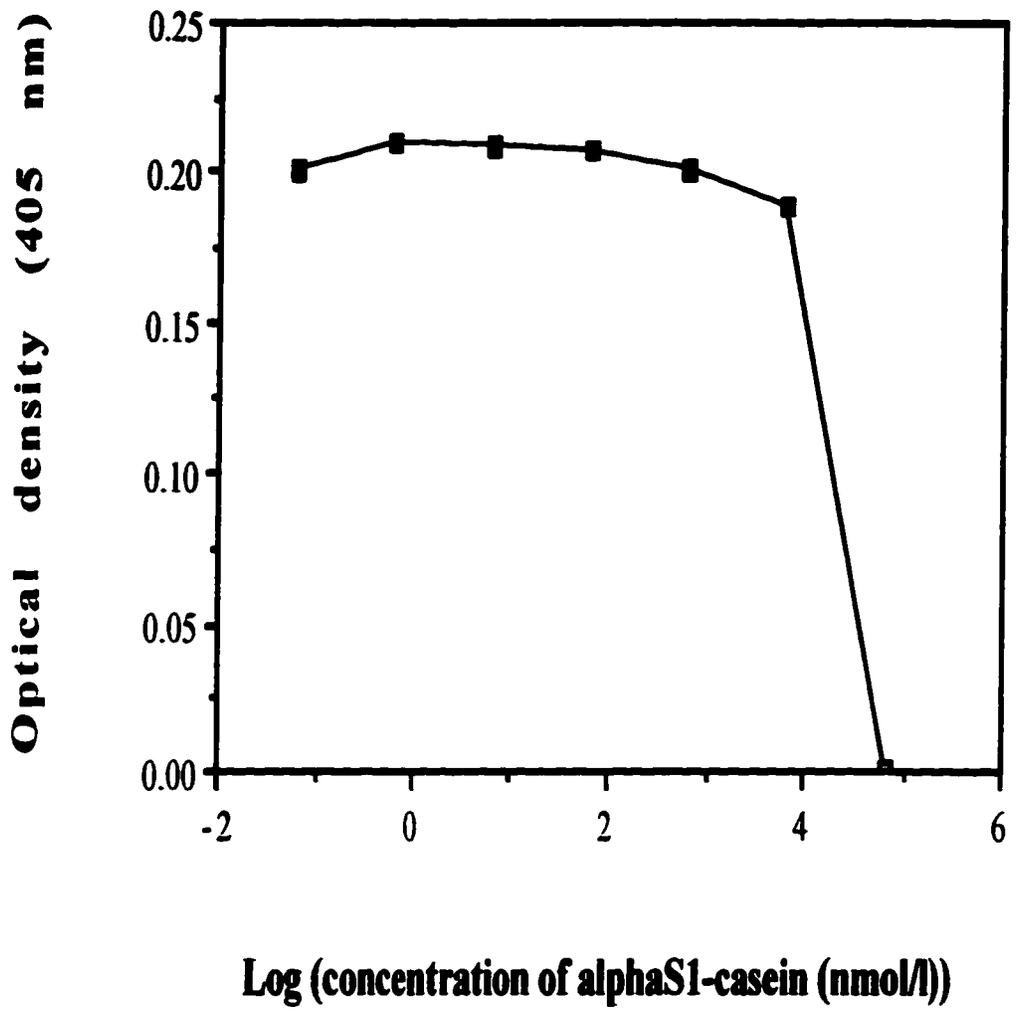

FIGURE 3.4: Indirect ELISA curve using α_{s1}-casein as competitor. Different concentrations of α_{s1}-casein are mixed with the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP are expressed as ELISA absorbances (OD 405 nm) at 1:1000 dilution. The data represents the logarithm of the concentration of α_{s1}-casein (nmol/l) plotted against absorbance. Values are the mean of 15 replicate measurements and the coefficients of variation (CV) were between 8.2 and 16.3%.

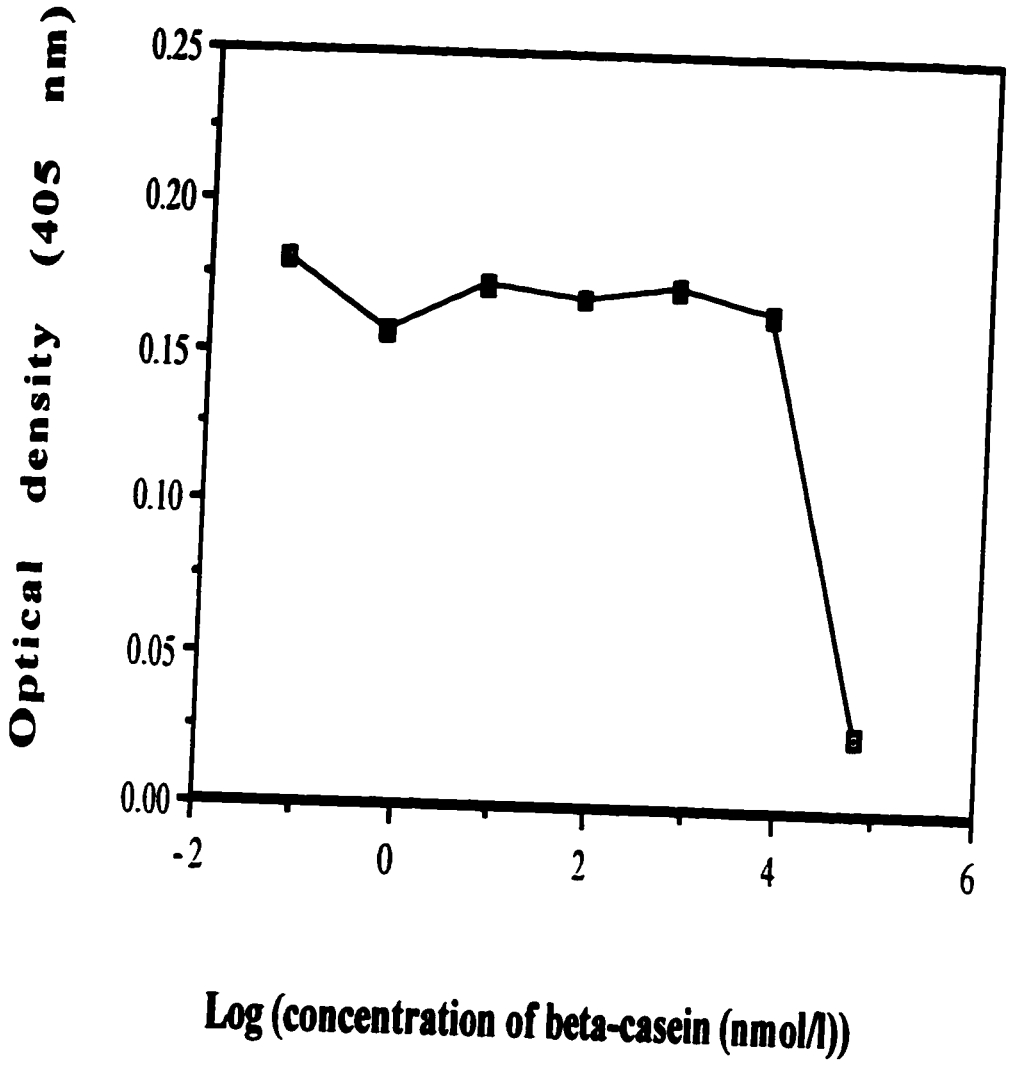

FIGURE 3.5: Indirect ELISA curve using β-casein as competitor. Different concentrations of β-casein are mixed with the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP are expressed as ELISA absorbances (OD 405 nm) at 1:1000 dilution. The data represents the logarithm of the concentration of β-casein (nmol/l) plotted against absorbance. Values are the mean of 15 replicate measurements and the coefficients of variation were between 9.8 and 16.2%.

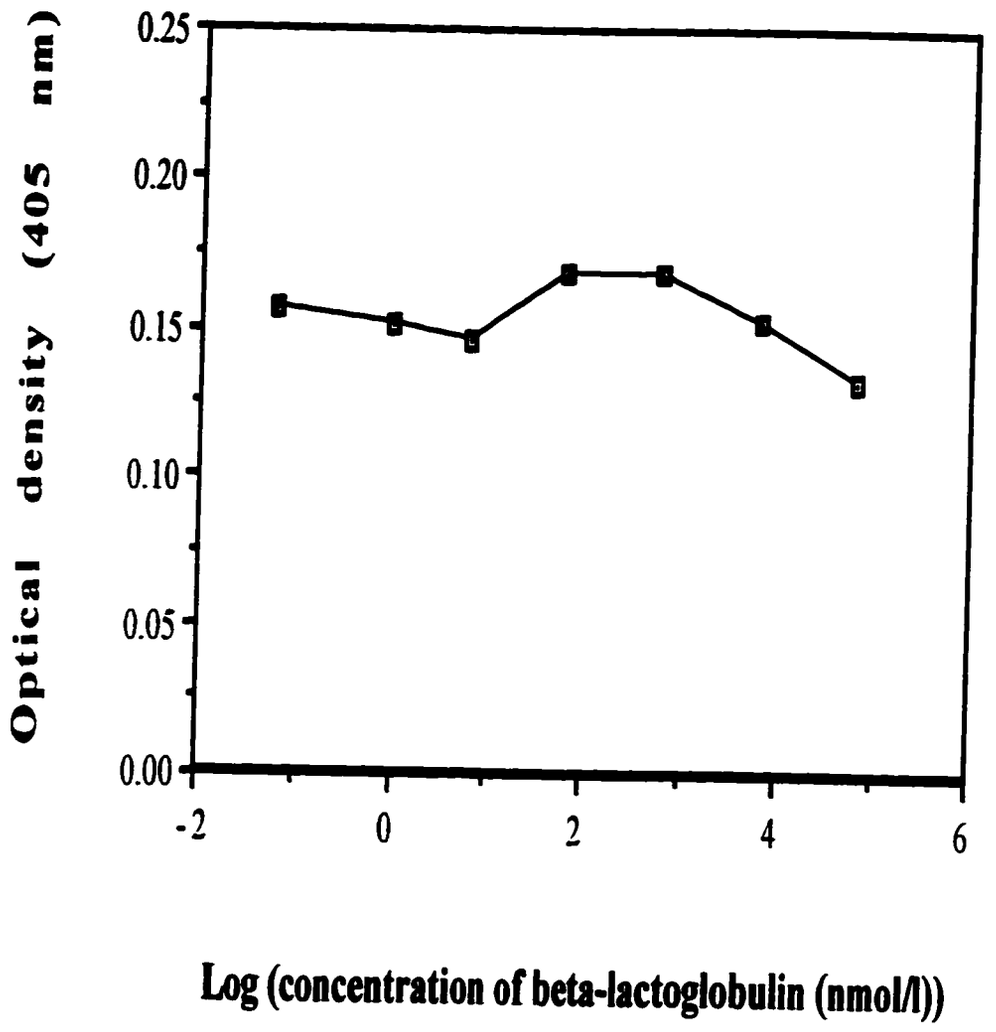

FIGURE 3.6: Indirect ELISA curve using β-lactoglobulin as competitor. Different concentrations of β-lactoglobulin are mixed with the IgY included in the wet precipitate (IgY-WP). Antibody levels in WP are expressed as ELISA absorbances (OD 405 nm) at 1:1000 dilution. The data represents the logarithm of the concentration of β-lactoglobulin (nmol/l) plotted against absorbance. Values are the mean of 15 replicate measurements and the coefficients of variation were between 9.9 and 17.3%.

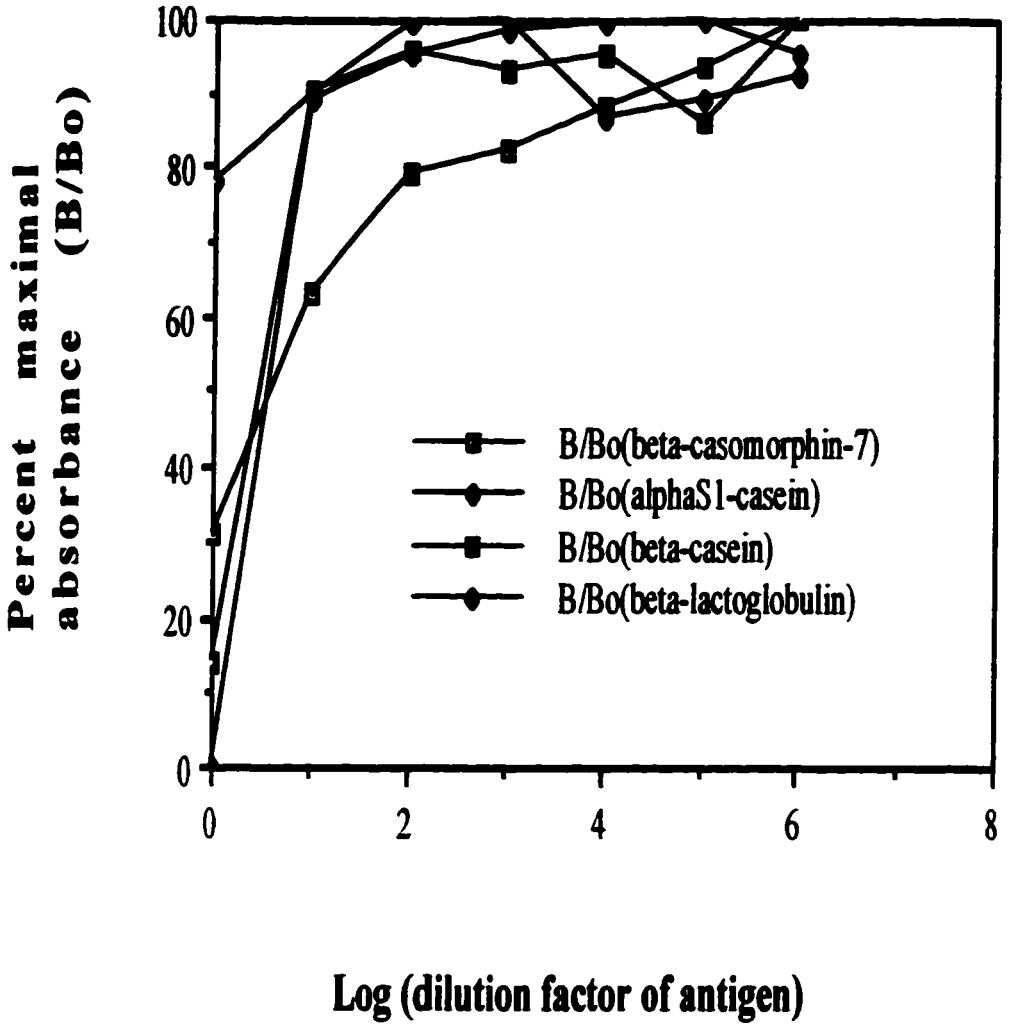

FIGURE 3.7: Indirect ELISA for β-casomorphin-7 in comparison with α_{s1}-casein, β-casein or β-lactoglobulin. The values (0 to 6) of the log of dilution correspond to concentrations of β-casomorphin-7 and the proteins in serial 10-fold dilutions from 62,500 to 0.0625 nmol/l.

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CHAPTER 4

CONCLUDING REMARKS AND RECOMMENDATIONS FOR FUTURE WORK

The objective of this study was the production of antibodies (IgY) in egg yolk against β-casomorphin-7 and the development of a reliable ELISA for the detection and quantification of the peptide in dairy products. For the first time the antibody production against β-casomorphin-7 has been achieved in egg yolks and the summary of the steps involved is presented in Figure 4.1.

Immunochemical analyses using enzyme immunoassays with anti-peptide antibodies developed in the egg yolks of laying hens have found many applications in food-related areas. This can be explained by the many advantages such techniques present over conventional methods, such as sensitivity, convenience, cost, ease and continuity of sample collection (1, 2).

It is important to note that not all hens may respond. In the present study six hens were used and one raised antibody against β-casomorphin-7. The antibody produced was further purified by precipitation with ammonium sulphate at 50% saturation. According to the literature (3), this is a convenient

way of preservation that does not interfere with the antibody activity. In the study conducted, loss of activity was observed after 9 months of storage at 0 °C. Based on this observation it is recommended to preserve the IgY-WP in lower temperatures, for example -20 °C. Another approach might be the examination of additional methods of purification and preservation for the IgY antibody. Such methods might include alcohol precipitation, ultrafiltration, and/or gel filtration.

The heat stability of the IgY antibody in the form of a wet precipitate was also studied and the results were similar to the ones obtained by Otani et al. (4) when he conducted a similar study with antibodies against α_{s1}-casein in the egg yolk. Almost 50 % of the antibody activity was retained when it was incubated at 70 °C for 20 min. These findings further support a potential role of antibodies from egg yolks as food ingredients, since they seem to be relatively resistant at heat treatments usually used in food processing.

An indirect ELISA mode was developed using the immunoreagent in the form of a wet precipitate. The standard curve obtained by indirect ELISA with β-casomorphin-7 was linear (r=-0.996) in the range 0.05-500 ng/ml and the detection limit was approximately 5 ng/ml.

Cross-reactivity of the IgY antibody with other milk proteins fractions was examined. No cross-reaction occurred with β-lactoglobulin and the antibody reacted with α_{s1}-casein and β-casein only when these two proteins were present at levels greater than 6,250 nmol/l. The absence of cross-reactivity at low concentrations can make the developed assay a useful tool for the

detection and quantitation in milk and other dairy products, since dilution of the examined sample will bring the concentrations of interest at low levels.

This is the first time that antibodies against such a small peptide (only 7 amino acids) are raised in the egg yolks of laying hens. Recently, Meisel (5) isolated IgY antibodies against β-casokinin-10, a decapeptide which corresponds to a bioactive sequence of bovine β-casein (5). There is no published information with regard to the level of β-casomorphin-7 in dairy products other than infant formulas. This target can be approached using the developed ELISA.

Three different strategies for the identification of bioactive peptides in milk proteins have been described in the literature up to date (6):

1) Isolation and characterization of bioactive peptides from *in vitro* digests of proteins.

2) Isolation and characterization of bioactive peptides from the gastrointestinal tract of experimental animals fed with a diet containing milk proteins;

3) Searching proteins for amino acid sequences similar to those known to be bioactive, followed by synthesis of these peptides and evaluation of their bioactive properties.

Figure 4.2 (adapted from reference 7) summarizes in a comprehensive way the various methodologies applied so far for the identification and characterization of bioactive peptides derived from milk proteins.

The developed ELISA could be a convenient tool for the detection and

measurement of β-casomorphin-7, as described in the above methodologies. One application might be for the detection and quantitation of the peptide in the intestinal contents of experimental animals after feeding trials.

Besides, in order to elucidate possible physiological significance and function of β-casomorphin-7 in the mammalian organism, large quantities of the peptide are needed. Therefore the IgY antibody raised against β-casomorphin-7 can be immobilized and used to recover β-casomorphin-7 from the β-casein enzymatic digests.

Figure 4.1. Flow diagram for the production of antibodies (IgY) in egg yolk against β-casomorphin-7.

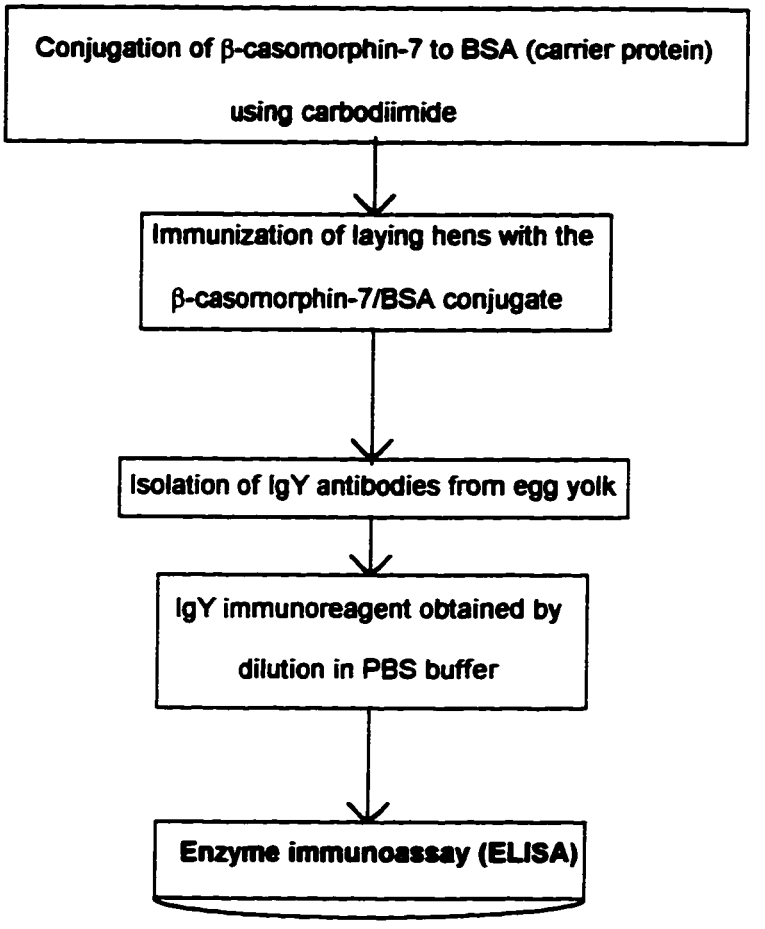
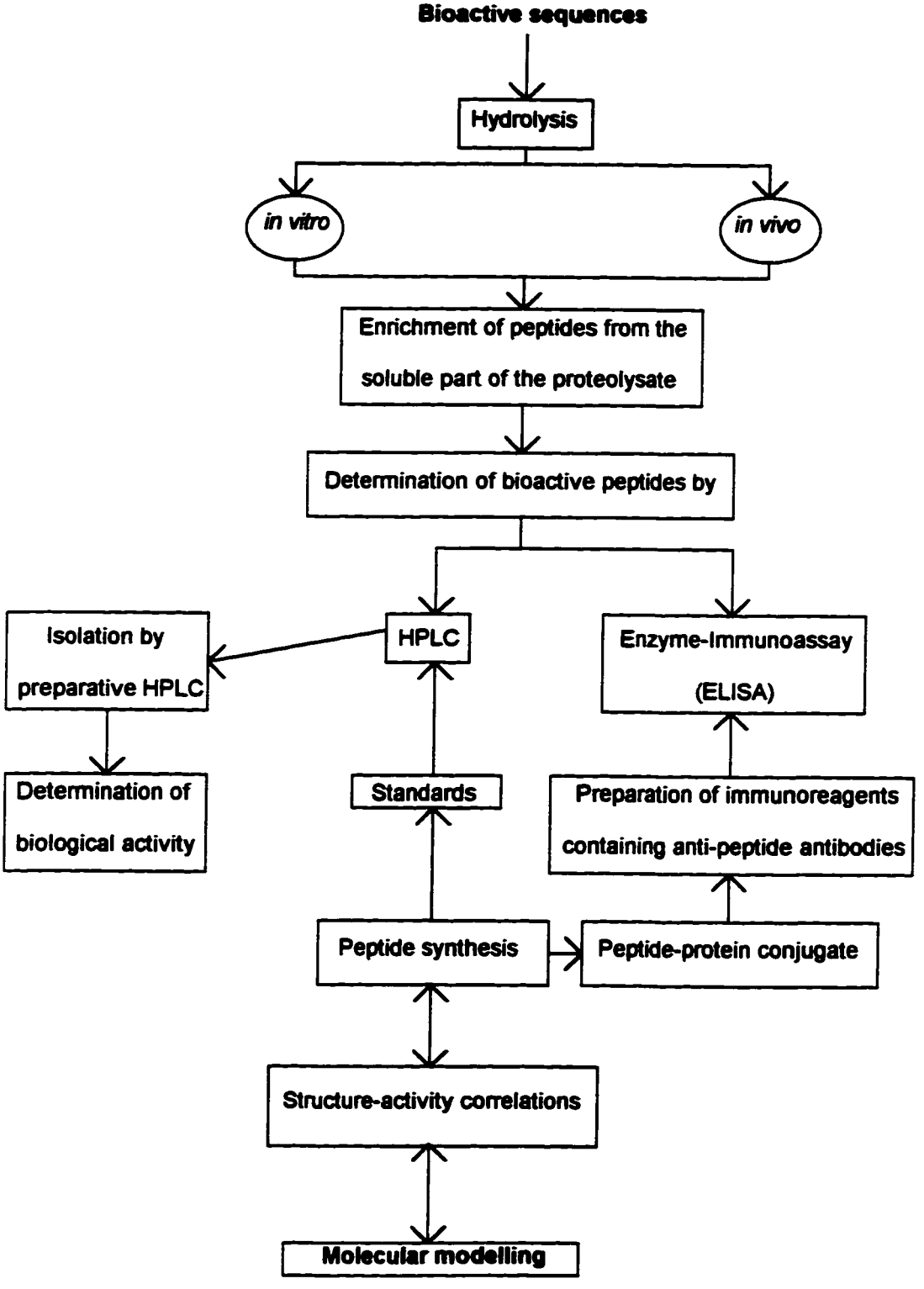


Figure 4.2. Flow diagram for the determination of bioactive sequences in milk proteins.



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